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Dr. Hanan Meyer
1911—1959

DR. HANAN (HANS) MEYER
1911–1959

A tragic accident cut short the life of Dr. Hanan Meyer who was just beginning to fulfil his aspirations.

Dr. Hanan (Hans) Meyer was born in Berlin on 27th January 1911. His father, Ziegfried (Arye) Meyer, who owned a printing press, died when Hanan was one year old. His mother, Johanna, raised him in Kostem, in the region of Pozan where he received his elementary education. After the Poles came to Pozan, he and his mother moved to Germany. In 1929 he received his high school certificate from the Reali Gymnasium in Praustadt. In 1934 he completed the course of pharmacy at the University of Breslau with distinction and in 1936 a course in chemistry.

That same year he moved to Israel. Since the Mandatory Government would not recognise a German degree in pharmacy, he undertook various kinds of menial labour but his great desire to work in research finally resulted in his employment by the Agricultural Research Station in Rehovot under Dr. Bondi where he gained his doctorate on a research work on animal nutrition.

Following the completion of this work, he was invited by Professor Fodor to serve as an assistant in the Department of Biological and Colloidal Chemistry at the Hebrew University of Jerusalem. He guided the biochemistry laboratory for students of chemistry and biology and lectured on selected topics in biochemistry. He was an able advisor and friend to his many students who greatly appreciated him.

Just a half-year before his death, Dr. Meyer's work gained recognition in his appointment to the position of Dean of the Faculty of Natural Sciences at the University of Tel Aviv.

Dr. Meyer had a severe critical sense, and just as he published his scientific works, he also kept many unpublished for further perfection. He also demanded the most from others, as he demanded of himself. Although this pedantry sometimes made it difficult for those working with him, his happy spirit and joy in creating pervaded his presence.

LIST OF PUBLICATIONS OF DR. HANAN MEYER

1. Chemical Composition and Digestibility of Palestinian Cattle Fodder, Agricultural Research Station, Rehovot, 1940, Bulletin No. 27.
2. The Digestibility of Citrus Feeds, 1941, *The Empire Journal of Agriculture*, **10**, 94.
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27. A Study of Nutrition Habits in Israel under Rationing Conditions, 1953, *ibid.*, 3, 15.
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HYDROLYTIC ACTIVITY OF CHOLINESTERASES AT LOW SUBSTRATE CONCENTRATIONS, CORRELATED WITH THE BIOLOGICAL EFFECTIVENESS OF CHOLINE ESTERS

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Department of Pharmacology, The Hebrew University–Hadassah Medical School, Jerusalem

ABSTRACT

By using isolated organs sensitive to choline esters, "rate of hydrolysis — substrate concentration" curves for true- and pseudo-ChE hydrolyzing the above choline esters, were extended to the low substrate concentration range in which graded biological responses can be obtained.

It is demonstrated that enzymatic hydrolytic rates and biological potency can be closely correlated. Therefore, it is suggested that the "receptor for choline esters" of any animal organ might be identical with either true- or pseudo- ChE.

Hydrolysis curves of cholinesterase (ChE) enzymes are usually determined in a concentration range above 1×10^{-3}M, occasionally 5×10^{-4}M, by manometric[2] or colorimetric[6] methods. The physiological concentration range of acetylcholine (ACh) is, however, much lower. Hydrolysis in this range can not be studied by the above methods.

This paper presents a biological method of determining enzymatic hydrolysis rates at low substrate concentrations. Determination by biological method is the oldest and it was used (before 1933) prior to characterization of the substrate specificity of true- and pseudo- ChE (1942).

The need for hydrolysis curves at low substrate concentrations in understanding the biological effectiveness of choline esters justifies the revival of the biological method. Our results show such curves obtained with true ChE of the electric organ of *Torpedo marmorata*[13] and the Electric eel (*Electrophorus electricus*), as well as with pseudo ChE of human plasma. These have not, apparently, been published until now.

ACh is a natural compound. It is widely held that the physiological role of ACh is that of "chemical transmission" of a nerve impulse between two nerve cells. It would be released from the nerve endings of the first cell at the junctional tissue (neuromuscular junction, synapses), reach by diffusion a specialized "receptor" of the next

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cell, where, by some physicochemical interaction with the "receptor", the nerve impulse would be propagated along the post junctional cell, which, however, may not be a nerve cell. The physiological role of ChE, abundantly present at synapses, would be to inactivate by hydrolysis the excess of released ACh.

In previous work[3,12,13,14,15,16] it was shown that the order of biological potencies of a homologous series of choline esters acting on different animal organs followed one of two patterns. In one, the order of activity resembled the order of hydrolysis by true ChE; in the other, it was similar to the order of hydrolysis by pseudo ChE. Generally, the more potent choline esters were found to be those which were more rapidly hydrolyzed by the corresponding ChE. We suggested that the receptor for "external" ACh acting on an animal organ should be identical with one of the ChE's.

We decided to pay special attention to the significance of the correlation between rate of hydrolysis by ChE's and biological potency of choline esters. In this paper an attempt is made to offer a rational interpretation of this correlation.

In this connection, it should be mentioned that Ahlquist[1] similarly described two potency patterns for the biological activity of adrenaline-like aryl-alkyl-amine derivatives which he ascribed to two related but different receptor structures, "α- and β- receptors".

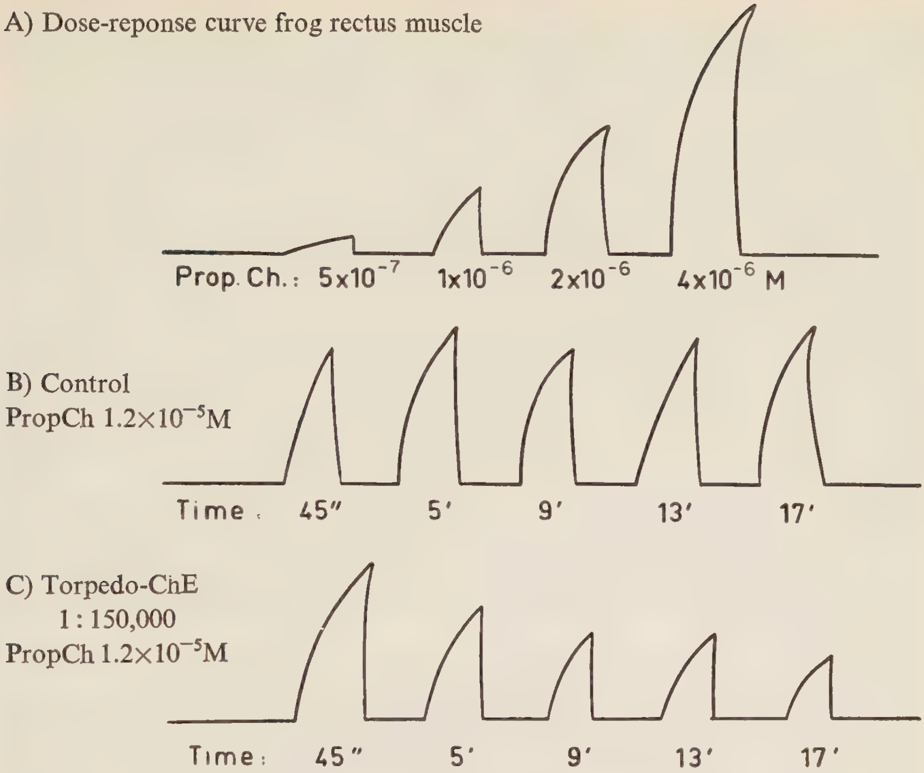
EXPERIMENTAL

Enzymatic hydrolysis measurements at low substrate concentrations

The method used for the determination of the rates of enzymatic hydrolysis at low substrate concentrations is illustrated in Figure 1a. Reaction mixtures made up of a buffered frog Ringer solution containing a suitable concentration of enzyme were incubated in a constant temperature water bath and the substrate added at the zero time of the reaction. Samples were taken at regular time intervals consistent with the recovery period of the "calibrated" isolated organ preparation. When using the frog rectus muscle, samples were taken once every 5 minutes and the enzyme concentration adjusted to cause a reasonable decrease in the contraction heights between samples (Figure 1b). By plotting the amount of choline ester still unhydrolyzed versus time, the tangent at zero time is obtained, which represents the initial velocity in the reaction mixture when the initial substrate concentration is still undiminished by the progressing hydrolysis.

As an example, in Figure 1 the reaction mixture contained propionylcholine (PropCh) 1.2×10^{-5}M. The enzyme used was Torpedo ChE at a dilution of 1:150,000 Part A shows the dose-response to PropCh of an uneserinized frog rectus muscle preparation; Part B shows the constancy of the responses to repeated equal doses; Part C shows responses to samples of the enzymatic reaction mixture, the samples being taken at intervals of 4–5 minutes.

The enzyme concentration used under these conditions is one tenth or less of that used in the manometric or colorimetric method. Therefore, this method can be applied to the determination of the cholinesterase activity of different excised tissues



A) Dose-reponse curve frog rectus muscle

B) Control
PropCh 1.2×10⁻⁵M

C) Torpedo-ChE
1 : 150,000
PropCh 1.2×10⁻⁵M

Figure 1a

Determination of enzymatic hydrolysis at low substrate concentrations.
A) The values for a dose-response curve with PropCh;
B) Repeated doses of PropCh 1.2×10^{-5}M, showing a reasonable constancy of responses;
C) Samples of an enzymatic reaction mixture containing Torpedo ChE 1:150,000 with PropCh 1.2×10^{-5}M in a buffered frog Ringer solution, taken at 45″, 5′, 9′, 13′, 17′.

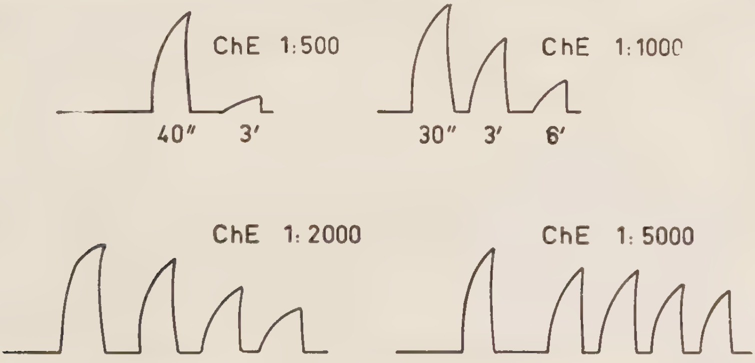


Figure 1b

Adjustment of enzyme concentration.

The reaction mixture contained a buffered solution of AChCl 200 μg/ml. The enzyme was EelChE The samples applied to the calibrated preparation were 0.003 ml. each; this volume contains 0.6 μg AChCl at O time of the reaction. The most appropriate reaction mixture contains ChE diluted 1:2000.

using modest amounts of tissue. This opens up new possibilities for ChE determinations on surviving tissues and blood. Also, as most organs contain a mixture of both types of enzyme, the ratio of true ChE to pseudo ChE could be determined.

When drawing the graph of the enzymatic hydrolysis curve, the hydrolysis rates had to be calculated for the same enzyme concentration. Therefore, when the concentration of the enzyme had to be changed during the experiment, upon proceeding from one concentration to the next, a check was also made to see whether twice or half the enzyme concentration still hydrolyzed twice or half in relation to the amount previously hydrolyzed. Thus it was found that the procedure of drawing curves from rate values calculated for the same enzyme concentration is justified. No further test for checking the linearity of the reaction rate — enzyme concentration relationship was used. The "rate of hydrolysis — substrate concentration" curves obtained (Figures 2,3 and 4) are representative, successfully completed experiments.

Cholinesterase preparations: Torpedo- and Electrophorus-electric organ ChE were prepared in this department, as described by Rothenberg and Nachmansohn (1947). Human plasma ChE was Edsall and Cohn's fraction IV.6.3, kindly provided by Dr. Ruth M. Flynn from the Department of Biological Chemistry of Harvard Medical School.

Choline esters: Commercial choline ester chlorides were used.

"*Calibrated*" *isolated organs*: The frog rectus muscle[5] was used for measuring small amounts of ACh, propionylcholine (PropCh), butyrylcholine (ButCh) and benzoylcholine (BenzCh). The guinea-pig intestine[5] was used for acetyl-β-methylcholine (MeCh) determinations.

RESULTS

1. *Hydrolysis with Torpedo true ChE*

a) Figure 2 shows the rate of enzymatic hydrolysis-substrate concentration curves obtained with Torpedo true ChE. The well-known order of hydrolytic rates at the optimum concentration of substrate ($pS_{\text{opt}} = -\log \text{conc}_{\text{opt}}$) is only maintained in the low concentration range. ACh is the best substrate, followed by PropCh, MeCh and ButCh, in that order.

b) BenzCh was not hydrolyzed at all by Torpedo true ChE. As seen in fig. 1, with PropCh at a concentration of 1.2×10^{-5}M, the enzyme concentration used was a 1:150,000 dilution of the original enzyme solution. An even greater dilution could be used for lower substrate concentrations. In order to see whether BenzCh could nevertheless be slightly hydrolyzed, we used the enzyme at a concentration 30,000 times higher than that mentioned for PropCh hydrolysis (1:5 dilution of the original solution). The results obtained showed that Torpedo true ChE did not hydrolyze BenzCh at all.

c) Carbamylcholine, a biologically very potent compound, was also found not to be attacked by true ChE at high enzyme concentrations similar to those used with BenzCh.

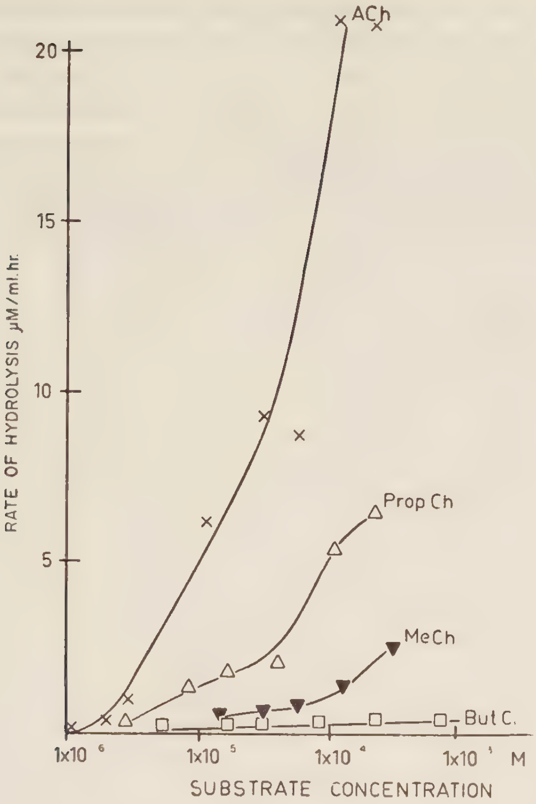


Figure 2
pS Activity Curves
Enzyme: Torpedo-cholinesterase
Substrates: Choline-esters

The rates of hydrolysis of choline esters by *Torpedo electric organ* true ChE as a function of the substrate concentration (the abscissa drawn on a logarithmic scale).

2. *Hydrolysis with Eel (Electrophorus electricus) true ChE*

The hydrolysis of choline esters with Eel ChE is seen in Figure 3. The curves for ACh and PropCh are almost identical with each other, as was also found in the region of optimum substrate concentration by others[4,9]. The order of hydrolytic rates is similar to that in the region of the pS_{opt}. The hydrolysis of PropCh appears equal to that of ACh; MeCh comes next, followed by ButCh. BenzCh was not hydrolyzed by this enzyme either.

3. *Hydrolysis with human plasma ChE*

Human plasma pseudo ChE activity at low substrate concentrations is seen in Figure 4. Only two points were determined for BenzCh. No real effort was made to extend the curve of BenzCh, although use of a calibrated eserinized leech muscle preparation would make this feasible. Only one point was determined for MeCh at a concentration just below 10^{-5}M. The rate of hydrolysis for the only MeCh concentration examined was quite low in comparison with that of other substrates; this low rate was as precisely determined as any other, being based on a record similar to that shown in Figure 1. It can be seen that ButCh remains the best substrate, even at low concentrations, followed by PropCh and then ACh.

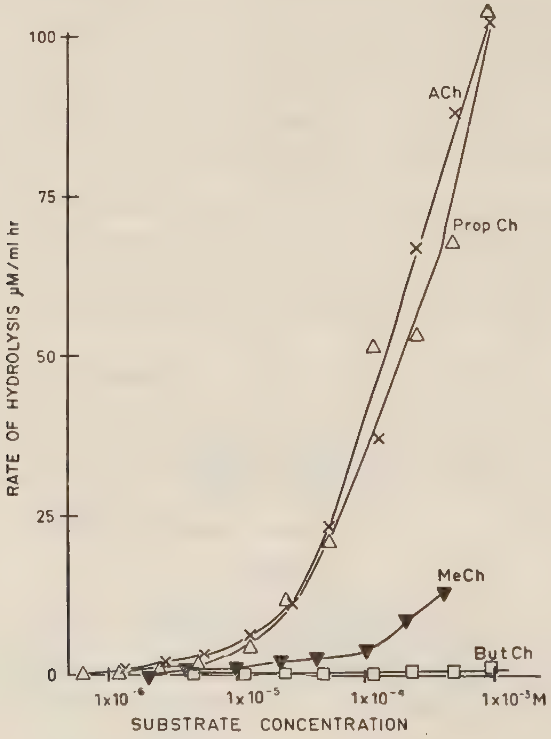


Figure 3
*p*S Activity Curves
Enzyme: Eel-cholinesterase
Substrates: choline-esters

The rates of hydrolysis of choline esters by *Eel electric organ* true ChE as a function of the substrate concentration (the abscissa drawn on a logarithmic scale).

DISCUSSION

1. One purpose of the presented experimental data was to find out whether, at substrate concentrations which come as close as possible to physiological concentrations, the order of rates of hydrolysis of the various choline esters undergoes any change. It has been demonstrated that the order of hydrolytic rates, previously studied only in concentration ranges of 10^{-3} — 10^{-2}M, is maintained in ranges 100

times or more lower. This is important biologically, and warranted clarification because of the slightly "anomalous" behavior of MeCh, as discussed below.

2. *Enzymatic hydrolysis versus biological potency*

a) As mentioned in the introduction, it was found in previous work that, when a homologous series of choline esters is used for a wide variety of isolated animal organs, their sequence of potency (actually of equipotent concentrations) is similar to the sequence of enzymatic hydrolytic rates by true- or pseudo- ChE. The reader is referred to Table I and II of Ref. (15) and to Table I and II of Ref. (16). *More potent choline esters seem to be those which are more rapidly hydrolyzed* by the respective type of ChE.

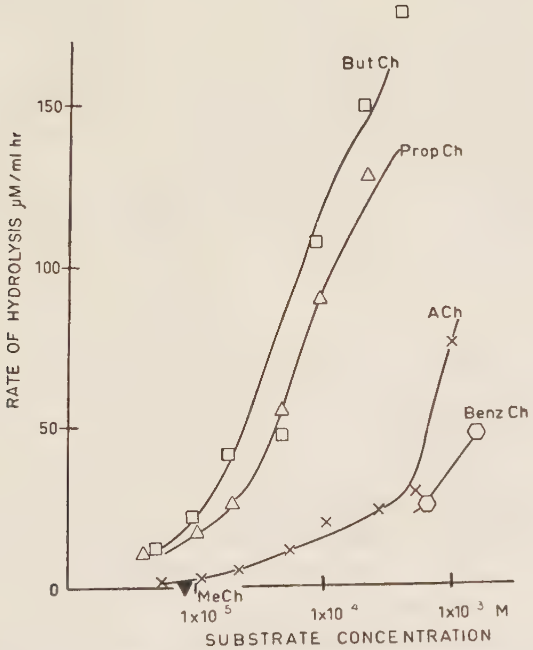


Figure 4
*p*S Activity Curves
Enzyme: human-plasma-cholinesterase
Substrates: choline-esters
The rates of hydrolysis of choline esters by *human plasma* pseudo ChE as a function of the substrate concentration (the abscissa drawn on a logarithmic scale).

b) We shall use here our hydrolysis curves for example for a piece of frog rectus muscle made to contract in vitro by these choline esters. Figure 5 shows the result of a typical experiment in which the potencies of ACh, PropCh, ButCh, BenzCh, MeCh are compared. The abscissa represents molar concentrations of choline esters on a logarithmic scale and the ordinate the height of contraction, in % of the maximal contraction height. The curves are "dose-response curves". A parallel line drawn to the abscissa from the 50 % value of the ordinate to intersect with each curve marks the equipotent concentrations of choline esters which, when applied to the orgen will cause the same contraction height.

The similarity of Figure 5 (biological potencies) and Figure 4 (hydrolysis by pseudo ChE) is so striking, that the ordinate in Figure 5 could be renamed "hydrolytic rate", instead of "height of contraction". Keeping in mind that the comparison is made between an enzymatic reaction in a defined homogenous watery medium and a complex biological response of a tissue, a better numerical agreement should not be expected. The parallel line now marks those substrate concentrations of different choline esters which are hydrolyzed at the same rate. Thus, if the function v = rate = k.E.S (where E and S stand for enzyme- resp. substrate concentration) represents hydrolysis by ChE's, then the condition for different choline esters to induce the same response (equipotency) can be described as

$$V_{\text{const}} = k_{\text{ACh}} \cdot E \cdot S = k_{\text{PropCh}} \cdot E \cdot S = \ldots$$

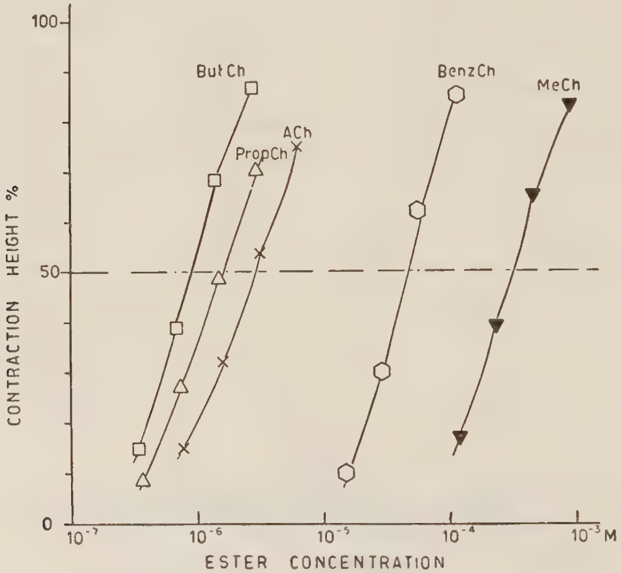


Figure 5
Relative potency of choline esters on the isolated frog rectus muscle.
The abscissa represents ester concentrations drawn on a logarithmic scale, the ordinate shows contraction heights expressed as percentage of the maximal contraction height. After F. Hobbiger, Student class experiment (1957) Ref. (7)

The conclusion from this interpretation is that 1) either the receptor for choline esters on the tissue is identical with ChE, or 2) the receptor reacts with these compounds similarly to the two ChE's in hydrolytic reactions*. If hydrolysis on the receptor spot is the common basis for the biological activity, then the criterion for equipotency must afford the same amount of reaction end product (in this case carboxylate, H^+ or choline-cation) per unit of time.

3. *MeCh acting on true ChE patterned organs*

From Table I[15] can be seen, that the order of biological potency is ACh > MeCh > PropCh > ButCh > BenzCh, whereas the order of hydrolytic rates at the PS_{opt} is ACh > PropCh > MeCh > ButCh > BenzCh. It was thought that the interchanged position in organ effects of PropCh and MeCh might be due to a corresponding reversal in the low substrate concentration range. However, the curves of hydrolysis presented here show that this is not the case, since the order of hydrolysis at PS_{opt} (3×10^{-3}M) is also maintained in the low concentration range. It can therefore be concluded that the above "anomaly" is not due to ChE. The subsequent examination of the whole homologous series of *β-methyl*choline esters (to supplement the already used MeCh) showed that within this second series of esters, enzymatic hydrolysis rate and biological activity maintain a similar relationship as within the choline series. We thus concluded that in the chain of biological events, some other step should account for the increased activity of MeCh. (see ref. (16)).

4. *The phenomenon of potentiation*

Although it is not proposed to discuss here so called "pharmacological evidences", it appears inevitable to mention data ostensibly conflicting with our account. Eserine, a strong inhibitor of ChE, in relatively small concentrations, "potentiates" the response of an organ to applied ACh — in other words, the same effect can be obtained with a smaller dose of ACh in the presence of eserine than in its absence. Therefore, eserine would be expected to inhibit "receptor-ChE" as well, unless permeability difficulties are present. This means that following our account, eserine should decrease sensitivity to ACh instead of increasing it, as it actually does. The answer to this can be found in another evidence derived from investigations on the role of plasma ChE. Plasma ChE has been called "transport ChE"[9] because any injected ACh meets plasma first, before reaching the organ on which an effect is observed. If plasma ChE is inhibited by eserine, partial destruction of ACh during transport is avoided. Therefore we suggest that "potentiating" concentrations of eserine always block "transport ChE" first, and "receptor ChE" only subsequently, due to differences in permeability, namely the easier access of ACh to "transport ChE" than to the less exposed "receptor ChE".

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* According to earlier claims, the frog rectus muscle contains exclusively true ChE. However, more recent work (see Jacob, J. and Dechavassine, M., 1956, *Exper.*, **12**, 434;1958, *Exper.*, **14**, 330 and Pecot-Dechavassine, M., 1958, *Comptes Rendus Acad. Sc.*, **247**, 2489.) and also our unpublished results indicate the presence of both enzymes. We have found no reports about purified enzyme preparations from this muscle in the literature.

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ENZYME SYSTEMS IN ADIPOSE TISSUE PARTICIPATING IN FATTY ACID ESTERIFICATION

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ABSTRACT

The mechanism of fatty acid esterification was studied in rat adipose tissue. The enzymes catalysing each step of esterification were present as follows:

A thiokinase catalysing the formation of higher acyl CoA compounds dependent upon CoA, ATP and Mg^{++} was found in the "acid particles" of rat adipose tissue extracts.

An "acyl-phorase", catalysing the enzymatic transfer of the acyl group of palmitoyl CoA to α-glycerophosphate, but not to free glycerol was present in the same "acid particle' fraction.

A phosphatidic acid phosphatase was demonstrated in the "large particle" fraction of rat adipose tissue extract.

Crude rat adipose tissue extract was found to contain a Ca^{++} activated lipase capable of hydrolysing its endogenous glycerides.

A scheme for the pathway of fatty acid esterification in adipose tissue is presented and discussed.

INTRODUCTJON

Radioactive 1-C^{14} labelled stearic or palmitic acid, when incubated with rat mesenteric adipose tissue in a suitable medium, is rapidly absorbed by the tissue and can be recovered in the tissue triglycerides (Shapiro *et al.* [1,2]). The esterification is especially high in the case of rats starved for several days and re-fed on a high carbohydrate diet 1–2 days prior to the experiment.

The present paper describes experiments carried out to elucidate the enzymatic mechanism of fatty acid esterification in adipose tissue and to find the possible precursor of the glycerol moiety of the glyceride esters.

MATERIALS AND METHODS

Animals

Normal rats of local strain weighing between 100 and 120 grams were used throughout the experiments. Where indicated the animals were starved, food being withheld for 1 or 2 days in case of the "starved rats" and for 5 days, followed by a 20 hr. period of re-feeding in case of the "re-fed rats".

Enzyme preparation

The rats were killed by a blow on the head and the mesenteric, testicular and retroperitoneal adipose tissue excised, placed in ice-cold isotonic KCl and cleaned from

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all extraneous matter. The tissue was then finely minced with scissors and homogenised in a high speed blendor in a mixture of 9 parts isotonic KCl and 1 part 0.2M phosphate or TRIS buffer at *p*H 7.3–7.4.

Fractionation of adipose tissue homogenate

The homogenised mixture was then centrifuged in a Servall centrifuge at 2,200 r.p.m. for 5 minutes and filtered through cotton wool. This filtrate was termed "adipose tissue crude extract". The latter was then re-centrifuged at 12,000 r.p.m. for 20 minutes and the "large particles" separated. The "supernatant" was filtered through cotton wool and stored 1 or 2 days in the freezer at –15°C. The supernatant was further fractionated on the day of experiment by quick thawing, bringing to *p*H 5.6–5.8 by means of 1N acetic or hydrochloric acid and centrifugation at 14,500 r.p.m. for 10 minutes. The resulting "acid supernatant" was decanted to the lastd rop andthe "acid particles" dispersed immediately in 0.2M phosphate. glycylglycine, or TRIS buffer *p*H 7.4–7.3. All operations were carried out in a cold room at temperatures 0–5°C.

Preparation of substrates

0.02M aqueous solutions of all even chain saturated fatty acids from C_2–C_{18}, as well as oleic acid, were brought to *p*H 8–9 by means of 0.1 N KOH.

CoA was obtained from Nutritional Biochemicals Corporation and ATP in the form of the dipotassium salt from the Sigma Chemicals Co. Palmitoyl CoA and radicactive palmitoyl CoA were prepared enzymatically according to Kornberg and Pricer[3]. Radioactive 1–C^{14} palmitic acid was obtained from the Radio-Chemical Centre, England.

Phosphatidic acid was prepared according to Kates[4] by the action of lecithinase C on pure egg lecithin prepared according to Pangborn[5].

Chemical and physical estimations

Esterified fatty acids were estimated according to Stern and Shapiro[6].

Inorganic phosphate was measured according to Fiske and Subbarow[7].

Paper chromatography of Palmitoyl CoA, and its reaction products was carried out according to Kornberg and Pricer[3].

Other chromatographic procedures employed included the separation of free palmitic acid from esterified palmitic acid using a MgO column (Borgstroem[8]).

Radioactivity measurements were made by means of a Tracerlab T.G.C. 2 thin mica end window counter.

RESULTS

Fatty acid activation

The supernatant fraction of adipose tissue homogenates was found to contain a thiokinase catalysing the reaction:

$$\mathrm{FA} + \mathrm{CoA} + \mathrm{ATP} \xrightleftharpoons{\mathrm{Mg}^{++}} \mathrm{FA} - \mathrm{CoA} + \mathrm{AMP} + \mathrm{PP}$$

which could be nearly completely concentrated in the acid particles therefrom. The enzyme activity was measured by the formation of acyl hydroxamate from the fatty acid thioester hydroxylamine being added to the reaction mixture according to Kornberg and Pricer[3]. Separation of the alcohol and water soluble hydroxamates was used to measure higher and lower fatty acid activation respectively. As illustrated in Table I the full system required CoA, ATP and Mg^{++}. Palmitic acid dependence was only partial, pointing to the presence of internal substrates. Phosphate ions were found to stimulate the activation. Fluoride caused no inhibition; lipoic acid had no significant effect.

TABLE I

Fatty acid activation dependence on cofactors

Enzyme	Cofactors	μE hydroxamate formed/2hrs/mg protein I	II	III
Supernatant before acidification	Full system	0.465	0.360	0.470
Acid particles in Tris buffer pH 7.4	Full system	0.942	0.848	
2 × concentrated	Full system			
	PO_4 buffer instead of TRIS, same pH	1.195	1.202	1.250
	CoA omitted	0.140		
	ATP omitted	0.078		
	Mg^{++} omitted	0.235		
	Palmitate omitted	0.554		
	Cysteine omitted	0.657		
	KF 25 μMoles added	0.870	0.795	
	after 24 hrs. dialysis against buffer			0.700
	after adsorptipn onto Norit			0.188
	Re-extracted from Norit			0.237
Acid supernatant	Full system		0.170	0.123

Unless otherwise stated the full system contained:
0.8 ml enzyme, 0.04 ml CoA (6mg/ml), 0.16 ml ATP (0.1M, neutral.), 0.15 ml $MgCl_2$ (0.154 M), 0.05 ml KPalmitate (0.02M), 0.10 ml TRIS buffer 0.5 M pH 7.4 (or where mentioned phosphate buffer 0.2M pH 7.4), 0.20 ml hydroxylamine hydrochloride 3M neutralized, 0.05 ml cysteine (0.4M) An equal volume of water was substituted where cofactors were omitted. Final volume 1.6 ml. Incubation time was 2 hrs. at 37°C in N_2.

The acid particle enzyme lost some of its activity upon dialysis overnight and nearly all of its activity upon adsorption onto Norit charcoal powder. Subsequent extraction of the charcoal with TRIS buffer failed to restore even a fraction of the original activity.

The effect of chain length on fatty acid activation is illustrated in Table II. The activating enzyme showed a pronounced specificity towards palmitic and oleic acid. Activation of lower fatty acids, measured by water soluble acyl hydroxamate formation (Kornberg and Pricer[3]) occurred only in fatty acids of $C_6 - C_{12}$ chain length and was negligible compared to the activation of the higher C_{16} fatty acids.

TABLE II

Effect of chain length on fatty acid activation

Substrate	Fatty acid activation, μE hydroxamate mg protein Experiment I	Experiment II
None	0.58	0.41
Acetate	0.58	0.32
Butyrate	0.54	0.30
Caproate	0.54	0.39
Caprylate	0.44	0.41
Caprate	0.64	0.41
Laurate	0.66	0.41
Myristate	0.68	0.42
Oleate	0.93	0.71
Palmitate	1.04	0.77
Stearate	0.50	0.53

Each tube contained 0.8 ml acid particle enzyme in phosphate buffer *p*H 7.4 (0.2M) and the constituents of the full system mentioned in Table I. Phosphate buffer was substituted for TRIS buffer and 0.05 ml of the respective fatty acid in 0.02M concentration for palmitate.
Incubation conditions as in Table I.

Acyl group transfer to "glycerol acceptor"

The reaction: Palmitoyl-CoA + acceptor → Palmitoyl acceptor + CoA was followed by the formation of acid soluble CoA, which was measured in a Beckman spectrophotometer (Model DU) at a wavelength of 256 mμ. Free glycerol and α-glycerophosphate (α-GP) were used as the glycerol acceptors of the fatty acid moiety of the palmitoyl CoA in the presence of adipose tissue enzyme. Deacylation of palmitoyl CoA occurred in all samples even without added acceptor as illustrated in Table III but α-glycerophosphate caused a definite additional liberation of CoA. Glycerol had no such effect, while Mg^{++} even caused a definite inhibition of free CoA production at higher concentrations.

TABLE III

Enzymatic liberation of CoA from palmitoyl CoA

Addition of palmitate acceptor	*CoA liberation* μMoles × 10^2
a.	
None	3.3
α-GP	7.8
α-GP + Mg^{++}(1.5 μMoles)	6.0
b.	
None	5.9
α-GP	9.8
α-GP + Mg^{++}(4.5 μMoles)	3.9
Glycerol	5.8

Each tube contained:
0.06 ml palmitoyl CoA (0.1 μMole), 0.04 ml glutathione (15mg ml), 0.08 ml acid particle enzyme in

glycylglycine buffer *p*H 7.5 (0.25M) and where stated, 0.04 ml α-GP (0.25M) or 0.04 ml glycerol (0.25M) or an equivalent amount of water. Total volume was 0.25 ml (Stansly[15]).
Incubation was carried out for 10 minutes at 25°C, and terminated by the addition of 1.8 ml 3% perchloric acid. Mixture was cooled in ice, centrifuged and the supernatant measured in Beckman spectrophotometer at λ256.

Radioactivity measurements of ether extracts of the reaction mixture before and after incubation showed an appreciable incorporation of the fatty acid label of palmitoyl CoA into an ether soluble reaction product in the presence of α-glycerophosphate (Table IV). By means of paper chromatography of these extracts and subsequent scanning of the chromatogram it was found that the radioactivity was located in an initial peak just after the start of the chromatogram, corresponding to the position of fatty acid glyceride esters. Radioactive palmitic acid was also usually found on the chromatogram having an Rf value of about 0.85. Unchanged palmitoyl CoA could not be extracted into ether and was always absent on the chromatogram. Chromatography on a MgO column yielded a radioactive eluate where α-G.P. was used as the acceptor proving the formation of a neutral labelled glyceride.

TABLE IV

Neutral fat production from 1-C^{14} *labelled palmitoyl CoA in presence of acceptor*

Acceptor	cts./min. in eluate after MgO column chromatography of ether soluble extract from reaction mixture	
None	55	30
α-GP	435	650
Glycerol	—	40

Phosphatidic acid phosphatase

The presence of phosphatidic acid phosphatase, could be demonstrated in adipose tissue by the liberation of inorganic phosphate from phosphatidic acid. This enzyme was more active in the "large particle" fraction of adipose tissue extract particularly when the activity was calculated per mg protein. α-Glycerophosphate also served as a substrate for this enzyme but only between 1/6 — 1/5 the amount of P was liberated in that case. The results are tabulated in Table V.

Adipose tissue lipase

Table VI illustrates the presence of Ca^{++} activated lipase in adipose tissue which catalysed the lipolysis of its endogenous glycerides. Most experiments were carried out with crude adipose extract but lipase activity was found also in both the supernatant and the "large particle" fraction separately.

TABLE V

Phosphatidic acid phosphatase in adipose tissue

Enzyme fraction	Substrate	Substrate concentration µMoles/ml	µMoles P liberated in 2 hrs.	Specific activity µMoles P/mg protein $\times 10^2$
NORMAL RATS				
Large particles	P.A.	3	1.42	—
Acid particles	P.A.	3	0.28	—
Large particles	P.A.	3.6	0.75	345
	α-GP	3	0.14	65
Large particles	P.A.	3.6	1.24	373
	P.A.	7.2	1.16	351
	P.A.	10.8	1.04	345
Supernatant	P.A.	6	0.35	53
	α-GP	2.5	0.08	12
Acid particles from supernatant	P.A.	7.2	1.08	20
	α-GP	3	0.21	4
STARVED-RE-FED RATS				
Large particles	P.A.	3.3	0.81	—
Large particles	P.A.	3	1.54	—
	α-GP	2.5	0.20	—
Same large particles 1 day old	P.A.	3	1.14	—
	α-GP	2.5	0.15	—
STARVED RATS				
Large particles	P.A.	3.6	0.68	406
	α-GP	3	0.12	70

Each tube contained:
0.1 ml particle suspension in TRIS buffer (or 0.2 ml of supernatant enzyme), 0.1 ml TRIS buffer 0.2M *p*H 7.3 and phosphatidic acid (P.A.) or α-glycerophosphate (α-GP) solution as indicated made up with water to 0.5 ml. Incubation for 2 hrs. at 37°C was terminated by the addition of 2 ml 10% trichloroacetic acid.

DISCUSSION

The study of the formation of fatty acid glycerides in adipose tissue is of special interest since this tissue is predominantly concerned with the synthesis and breakdown of fat. The demonstration in adipose tissue of all the reaction steps involved in glyceride synthesis in liver (Weiss and Kennedy[9], Stein and Shapiro[10], Stein, Tietz and Shapiro[11]) points to the following sequence of reaction:

1. $\text{Fatty acid} + \text{CoA} + \text{ATP} \xrightleftharpoons[\text{Mg}^{++}]{\text{thiokinase}} \text{Fatty acid-CoA} + \text{AMP} + \text{PP}$

2. $\text{Fatty acid-CoA} + \alpha\text{-glycerophosphate} \xrightarrow[\text{phorase}]{\text{Acyl-}} \text{Phosphatidic acid} + \text{CoA}$

3. $\text{Phosphatidic acid} \xrightarrow[\text{phosphatase}]{\text{phosphatidic acid}} \text{Diglyceride} + \text{P-inorganic}$

4. $$\begin{tikzcd} \text{Diglyceride} + \text{Fatty acid CoA} \arrow[r, "\text{Acyl-}", "\text{phorase (?)}"'] & \text{Triglyceride} + \text{CoA} \arrow[l, bend left=40, "\text{Lipase}"', "\text{Ca}^{++}"] \end{tikzcd}$$

TABLE VI

Lipase activity in adipose tissue

Type of rat	Enzyme	Incubation time (hrs.) at 37°C	% Lipolysis	Ca^{++}
Normal	Crude extract	2	55.4	+
Normal	Crude extract	2	25.9	—
Starved (2 days)	Crude extract	2	27.2	+
Starved (2 days)	Crude extract	2	6.3	—
Starveld Re-Fed Rats	Crude extract	$1\frac{1}{2}$	54.5	+
	Crude extract	$1\frac{1}{2}$	40.0	—
	Supernatant	$1\frac{1}{2}$	33.4	+
	Supernatant	$1\frac{1}{2}$	15.4	—
	Supernatant + added fat	$1\frac{1}{2}$	23.3	+
	Supernatant + added fat	$1\frac{1}{2}$	11.6	—
	Large particles	$1\frac{1}{2}$	36.0	+
	Large particles	$1\frac{1}{2}$	19.3	—

Each tube contained:
0.5 ml enzyme, 0.2 ml phosphate buffer pH 7.4 (0.2M), 0.06 ml $CaCl_2$ (0.05M) or equivalent amount of H_2O.
Incubation was terminated by the addition of alcohol ether (3:1), the mixture diluted to a known volume and aliquots tested for ester content by the hydroxylamine test.

Higher fatty acid metabolism seems to be a special feature of adipose tissue (Shapiro et al.[1,2]) and in this connection it is of interest to note that the presence of a thiokinase active only for those acids could be demonstrated.

In the esterification process the labelled fatty acid group of palmitoyl CoA was found to be transfered enzymatically to α-glycerophosphate but not to glycerol, and was partially converted into an ether soluble radioactive compound, with the liberation of CoA. The high degree of deacylation of palmitoyl CoA under our experimental conditions rather interfered with the formation of labelled palmitoyl —

"glycerol-acceptor"; nevertheless a radioactive ether soluble compound was obtained in the presence of α-glycerophosphate, chromatographically distinct from palmitic acid and palmitoyl CoA. This compound, which was not formed when labelled palmitic acid was substituted for labelled palmitoyl CoA passed a MgO column. Therefore it does not seem to be identical with phosphatidic acid but was more probably a neutral glyceride. Complete chemical identification was not possible as it was present only in minute amounts.

The presence of phosphatidic acid phosphatase in the large particle as well as the supernatant fraction of adipose tissue extract provides the clue to the third step in the esterification of fatty acids, namely the dephosphorylation of phosphatidic acid to a diglyceride. This enzyme is much less active than its analogue in liver (Garcia-Pineda *et. al.*[12]).

A lipase catalysed hydrolysis of triglycerides, activated by Ca^{++} ions was also shown to be present in adipose tissue. The resulting di- and monoglycerides as well as the diglyceride formed on the dephosphorylation of phosphatidic acid may now interact with excess palmitoyl CoA to form triglyceride in the final step of fatty acid esterification. Proof for this process is, however, still outstanding.

The two alternative routes to diglyceride formation, an assimilative and a dissimilative one, may take place simultaneously or successively, probably depending on the nutritional state of the rat. During starvation, mono- and diglycerides accumulate in adipose tissue (Wadstroem[13]) probably due to increased lipolysis.

More prolonged starvation may reduce some of the glycerides to glycerol, which is subsequently utilized as carbohydrate. In the period of re-feeding a high carbohydrate diet, the resulting increased glucose metabolism may furnish new glycerol precursors in the form of α-glycerophosphate, which can then act as acyl acceptor.

Degradation studies carried out on radioactive triglycerides, newly formed from $1\text{–}C^{14}$ labelled palmitate by adipose tissue *in vitro* point to a total synthesis of triglycerides when the tissue was derived from rats previously starved for a long period and re-fed prior to the experiment (Shapiro et al.[14]).

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PROTEOLYTIC ACTIVITY OF CHICKS' INTESTINES

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ABSTRACT

A method for enzyme preparation from the intestine content (chyme) of chicks is described and data on the proteolytic activity of growing chicks' intestine are given.

Total enzyme activity was found to be dependent upon amount of chyme. The activities per unit chyme weight were approximately the same in all preparations and not correlated to age and interval after eating.

INTRODUCTION

It is known[1] that the main proteolytic digestion of the chick takes place in the small intestine.

Peptic digestion in the gizzard is rather weak and it is likely that the proteolytic enzymes of the pancreas are mainly responsible for proteolysis. However, little is known about tryptic digestion of chicks and little qualitative data are found in the literature[2].

This work was undertaken in order to obtain: 1) a reliable method for quantitative determination of the intestinal proteolysis in the chick; 2) experimental data about proteolysis in chicks fed a practical ration, with regard to the effect of age and intervals after feeding.

The results obtained could be useful in studying the mode of action of any factor upon chick digestion; e.g. the effect of raw soybean meal on chick tryptic digestion is at present being investigated in this laboratory.

EXPERIMENTAL

a) *Chicks.* 100 White Leghorn chicks one day-old were fed a commercial chick mash containing 21.0% crude protein, twice daily: from 7–9 A.M. and from 1–3 P.M. Their proteolytic activity was tested from the age of 2–7 weeks and at different intervals after feeding. Three such experiments were carried out.

b) *Enzyme preparation.* The chicks were killed by rapid bleeding and the small intestine, from the posterior end of the duodenum to the juncture with the caeca was immediately removed. The duodenum was not included in the preparation for the following reasons: 1) the pancreatic duct empties into the distal end of the duodenum, 2) ingested food was usually not found in the duodenum.

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The chyme was squeezed out and the mucus separated. The resulting material of three chicks was weighed and then homogenized with 10 volumes of distilled water in an Ultra-Turrax apparatus.

The suspension was immediately used for the determination of proteolytic activity.

c) *Determination of the proteolytic activity*: Kunitz's procedure for trypsin determination, described by Colowick and Kaplan[3] was followed with only slight modification: the enzyme suspension (0.1–1.0 ml) was brought to 2 ml with the phosphate buffer, *p*H 7.6. After a reaction of 20 minutes at 37°C with 1 ml 1% casein, 5 ml of 10% trichloroacetic acid (T.C.A.) were added. After 1 hour, the samples were centrifuged at 5000 rpm and filtered. Unclear solutions were recentrifuged in a Superspeed Servall centrifuge (about 8000 rpm). The optical density of the supernatant was read at 280 mμ in a Beckman DU Spectrophotometer with the reagent mixture as blank.

The readings were corrected for the values of blanks, which were prepared in the same way as the reaction, but in different order: the T.C.A. was added to buffered enzyme before the addition of casein.

RESULTS AND DISCUSSION

a) *Preparation and homogenity of samples*

As mentioned in the Experimental, the enzyme preparation was used in suspension, without centrifugation. The reason for this was that even mild centrifugation (2000 rpm, 10 minutes) always lowered the activity of the preparation.

Inferior activities were also obtained whenever the preparation contained a marked quantity of intestinal mucus. The mucus, when tested separately, showed low and non-characteristic enzyme curves, and was therefore eliminated from the preparation. A comparison of typical activities of suspension, supernatant and mucus is shown in Figure 1.

The homogenity of the preparation was proved by measuring the activity of triplicates of four different volumes. A low coefficient of variation was obtained (Table 1).

TABLE I

Optical densities of triplicates.

ml of enzyme preparation	at 280 mμ	average	coef. of var. in %
0.1	0.220 0.200 0.200	0.207	5.5
0.2	0.430 0.415 0.440	0.428	2.9
0.3	0.552 0.515 0.590	0.552	6.7
0.5	0.690 0.775 0.735	0.733	5.8

The variability of individual chick activities is presented in Figure 2.

The coefficient of variation between individuals was found to be the lowest (about 13%) with 0.5 ml of preparation, i.e. about 50 mg of intestinal content. At this point all the preparations approached maximum activity. Above 0.5 ml the curves usually remained at the same level or even tended to decline.

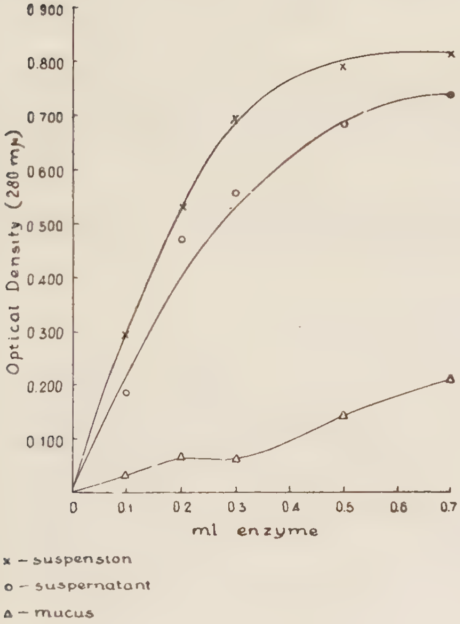

Figure 1
Activity curves of suspension, supernatant and mucus. Each point represents the average of 3 experiments, with 3 chicks each (9 animals).

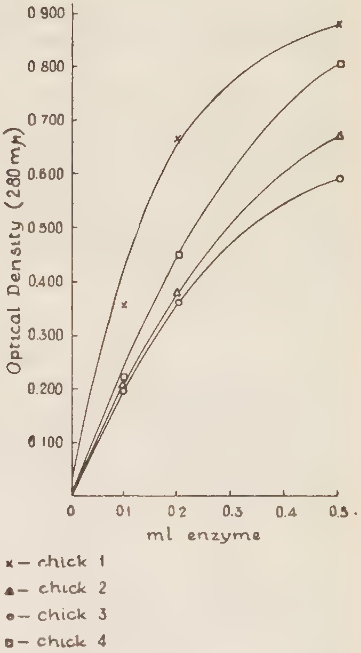

Figure 2
The enzyme curves of individuals

b) *Measurement of proteolytic activity.*

Samples of intestinal content were tested at different intervals after feeding, in chicks 2–7 weeks old. The enzymatic activity of samples collected early in the morning, after a fast of 17 hours, was irregular and always much lower than that of birds freshly fed. From one to six hours after feeding began, regular enzyme curves, very similar in slope and height, were obtained. As the 0.5 ml of preparation showed the lowest coefficient of variation between individuals, activity at this point was chosen for presentation of the data. The values of activity for the 0.5 ml of the preparations 2, 4 and 6 hrs after the beginning of eating are presented in Table IIa. The results are expressed in optical density at 280 mμ.

TABLE IIa

*Proteolytic activity at different ages and hours after eating**

Age (weeks) Hrs after eating	2	3	4 O. D. at 280 mμ	6	7
2	0.840	0.781	0.758	0.625	0.850
4	0.714	0.707	0.725	0.787	0.775
6	0.787	0.742	0.714	0.764	0.895

* Average of three experiments (9 chicks)

No appreciable difference in activity was observed between ages and hours after eating, when the same concentrations of chyme were compared (Table IIa) and no correlation was found between the above enzyme activity and the intestinal content nor its percentage of dry matter. (Table IIb).

However, as the volume of preparation depended on chyme weight, *total* enzyme amount was proportional to the amount of chyme present.

TABLE II b

*Intestinal content (g) and dry matter (%)**

Age (weeks)	2		3		4		6		7	
Hrs after eating	g cont	% dry matter	g cont	% d. m.	g cont.	% d. m.	g cont.	% d. m.	g cont.	% d. m.
2	4.0	16.2	9.9	18.3	8.3	19.1	17.6	17.7	13.0	19.9
4	4.6	18.1	9.6	17.8	12.5	17.2	16.9	16.9	12.8	18.4
6	4.4	13.5	5.8	15.1	8.4	18.5	11.4	16.4	9.6	20.7

* See foot-note to table IIa

In order to obtain an idea of the actual proteolytic capacity of the enzyme in the intestine the following calculation can be made: the average optical density obtained (Table IIa) was 0.760, which corresponds to 5 μeq of tyrosine (a standard tyrosine solution gave under the same conditions an O.D. of 0.151 for each μeq).

One ml (10 mg) of the casein solution digested by trypsin (no precipitation with T.C.A.) gave an O.D. corresponding to 8 μeq typrosine.

From the above numbers and on the basis of total volumes of intestine preparation – about 40 ml in young chicks and up to 170 ml for older ones — it could be calculated that the enzymes present in the small intestine are able to digest approximately from 0.5 g to 2.0 g casein within 20 minutes.

According to accepted physiological descriptions, the pancreas secretes enzymes which blend with the food whenever it passes through the duodenum. It seems that the total amount of proteolytic enzymes present in chyme depends only on the amount of food passing into the intestine. Therefore, in preparations based on the same chyme concentration, a similar enzyme activity was found without any correlation to dry matter of the content, time after eating and age of the chick.

ACKNOWLEDGMENT

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CELLBOUND PENICILLINASE AND EXOPENICILLINASE IN THE γ-STATE

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ABSTRACT

The cellbound γ-penicillinase formed by *Bacillus cereus* differs from the exopenicillinase liberated by the same organism in its antigenic properties and in its sensitivity to iodine. Exposure to urea confers on the exopenicillinase properties similar to those of the cellbound penicillinase. The latter has been purified and its reactivity with iodine compared with that of urea-modified exopenicillinase. In both cases the iodine sensitivity decreases following contact with the substrate. Quantitative differences in the reactivity with iodine and in response to the substrate have been established, and their significance discussed.

INTRODUCTION

A large proportion of penicillinase formed by *Bacillus cereus* is liberated into the medium. This "exopenicillinase" fraction accounts for about 90 per cent of penicillinase activity found in the culture. A part of the remaining enzyme is found adsorbed on the cells and it can be eluted with strong salt solutions. It is believed to be identical with the exopenicillinase[1]. Another part of the cell-bound enzyme (which accounts for 5–7 per cent of the activity of the culture) cannot be solubilized unless the cells are extensively disintegrated. This fraction of cell-bound penicillinase has been shown to differ antigenically from the exopenicillinase, and it has been designated as cell-bound γ-penicillinase[1] (the abbreviation CB-γ-penicillinase will be used).

Pollock[1] has compared the two fractions and found them to be identical in most of the other properties studied, including substrate specificity, Michaelis constant and sedimentation rate. However, CB-γ-penicillinase has been found to be inactivated by iodine concentrations which have no effect on the activity of the exopenicillinase.

Further studies have shown that under certain specified conditions, exopenicillinase may change its antigenic properties and become sensitive to iodine[2]. The change which is essentially reversible, has been termed a change from the α-state to γ-state. Thus exopenicillinase in the γ-state is very similar to CB-γ-penicillinase, except for fact it can revert to the α-state, while no such reversion has been observed in CB-γ-penicillinase.

The present work is concerned with comparing some properties of CB-γ-penicillinase with those of exopenicillinase in the γ-state.

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MATERIALS

Source of penicillinase

A strain of *Bacillus cereus* (NRRL 569/H) which produces penicillinase constitutively was inoculated into a liquid medium prepared according to Kogut et al[3] and dispensed in 500 ml portions in 2 l Erlenmeyer flasks. The flasks were incubated at 35°C on a rotary type shaker for about 10 hours. The cultures were centrifuged, the cells were used for preparing CB-γ-penicillinase, while the supernatant served as a source for exopenicillinase.

CB-γ-penicillinase. The cells harvested from the above cultures were washed 3 times with 1 M NaCl in 0.1 M Na-citrate. The supernatant of the third washing was assayed and found free of penicillinase activity, while the washed cells were found to contain a high proportion of iodine sensitive penicillinase. The cells were then suspended in 10 ml of twice distilled water and disrupted in a Raytheon 9 Kc sonic oscillator for 30 minutes. The sonicated material was centrifuged at 10,000 rpm for 20 minutes and the sediment discarded. The supernatant of the sonic extract was found to contain 268 units of penicillinase per mg of protein. It was dialysed for 2 hrs at + 2°C against 20 volumes of 0.001 M phosphate buffer *p*H 6.5. A freshly prepared 10% solution of protamin-sulphate was adjusted to *p*H 6.5 and added to the dialysed material so as to give a final concentration of 2 mg protamin-sulphate per ml. The heavy precipitate which appeared almost instantaneously was spun off, and the supernatant was further purified and concentrated by the adsorption and elution procedure described elsewhere[4]. The penicillinase activity of the purified preparation was 2500 units per mg protein.

Exopenicillinase. The supernatant of the 10 hour old culture (see above) has been concentrated and purified by selective adsorption and elution[4]. The specific activity of the preparation used was close to that found for crystalline penicillinase[3].

RESULTS

The iodine sensitivity of CB-γ-penicillinase

The sensitivity of CB-γ-penicillinase to iodine has been measured by assaying the enzyme in the presence of varying concentrations of iodine by the otherwise unmodified iodometric procedure described elsewhere[2,4]. In Figure 1 the surviving activity is compared to that of exopenicillinase assayed in 3.5 M urea at the corresponding concentrations of iodine. It has been shown that exposure of exopenicillinase to urea causes an α to γ shift[5]. The survival of exopenicillinase activity in urea is inversely proportional to the concentration of iodine within the range tested, whereas if urea is replaced by 0.5% gelatin, as in the standard assay procedure[2], the activity is independent of the iodine concentration[6]. Thus curve A, Figure 1, represents the iodine sensitivity of urea induced γ-state of exopenicillinase while curve B (Figure 1) represents the iodine sensitivity of CB-γ-penicillinase.

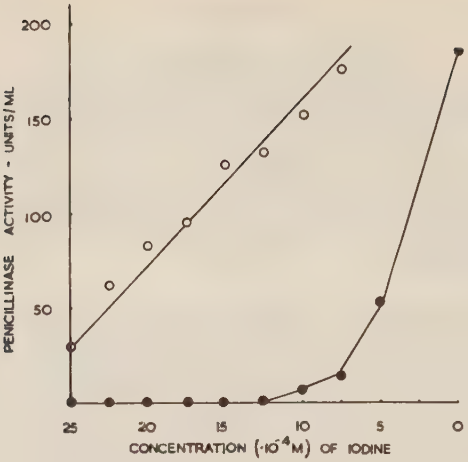

Figure 1
Survival of penicillinase activity as a function of the initial iodine concentration.
(A) 0—0 exopenicillinase in the γ-state
(B) ●—● CB-γ-penicillinase

It will be noted that CB-γ-penicillinase is much more sensitive to intermediate iodine concentrations than the urea induced γ-state penicillinase. Thus at the concentration of 12.5×10^{-4}M iodine CB-γ-penicillinase is completely inactivated, while the activity of exopenicillinase in urea is largely spared.

Iodine sensitivity following contact with the substrate

Previous tests[6] have shown that the iodine sensitivity of exopenicillinase in urea decreases in the course of its interaction with the substrate. The test for change of susceptibility of penicillinase to iodination following exposure to the substrate, has been described elsewhere[6]. It is based on the iodometric assay of penicillinase[2], but whereas in the standard assay procedure the reaction is started in the presence of iodine, here iodine is introduced at specified time intervals after the reaction has begun. While the delay in addition of iodine does not affect the activity of penicillinase in the α-state[6] it is decisive for the survival of penicillinase activity in urea.

The delay in addition of iodine is even more critical for the survival of CB-γ-penicillinase — as shown in Figure 2. It will be realized that the concentration of iodine to which the enzyme is exposed decreases with the delay. This is because before the addition of iodine the enzyme produces an amount of penicilloic acid proportional to the time of delay; penicilloic acid combines instantaneously with the added iodine and reduces its effective concentration to a value inversely proportional to the time of delay.

The concentration of the enzyme used in these experiments was adjusted so that the amount of penicilloic acid formed in 30 sec would be just sufficient to combine with

2.5×10^{-4} M iodine. It has been shown in a previous communication[6] that the rate of formation of penicilloic acid is linear and that the combination of iodine with the penicilloic acid formed is practically instantaneous. Hence the actual iodine concentration to which the enzyme is exposed at a specified time in the course of the reaction can be calculated (Table I, columns A, B). The activity of CB-γ-penicillinase exposed simultaneously to the substrate and to a given concentration of iodine, assayed as shown in the previous section (see also Figure 1), is given under column C in Table I. This is the activity which is to be expected following delay in adding iodine, on the basis of the resulting iodine content of the assay system. Column D gives the observed activity following such delay. As already shown[6] any discrepancy between the expected and observed values under the conditions described reflects the effect of preincubation of the enzyme with the substrate.

TABLE I

The effect of preincubation with the substrate on the subsequent activity of CB- γ -penicillinase

A Time before iodine addition (min)	B Iodine concentration $\times 10^{-4}$ M	C Expected survival of activity (units/ml)	D Observed survival of activity (units/ml)
0.0	25.0	0	0
0.5	22.5	0	0
1.0	20.0	0	0
1.5	17.5	0	0
2.0	15.0	0	10.3
2.5	12.5	0	16.4
3.0	10.0	7.3	42.5
3.5	7.5	14.1	65.5
4.0	5.0	52.5	112.0

Comparison of columns C and D in Table I indicates a definite increase in the iodine resistance of CB-γ-penicillinase following contact with the substrate. The substrate induced change in iodine-reactivity is here much smaller than in the case of exopenicillinase in the γ-state (cf. Figure 2).

The effect of electrolytes

The change from α-state to γ-state can be prevented or even reversed by M NaCl. This phenomenon is demonstrated in Figure 3. It appears to be due to a general electrostatic effect shared by other electrolytes[4]. As can be seen from Figure 3, there is no indication of any similar effect of the electrolyte on CB-γ-penicillinase.

The apparent irreversibility of CB-γ-penicillinase suggested that the enzyme here may be attached to an unknown cell component, possibly through hydrogen bonds. Such possibility could perhaps be tested in the present system. Exposure of CB-γ-penicillinase to 3.5 M urea might be expected to break such intermolecular bonds, but it would prevent formation of the intramolecular hydrogen bonds believed to

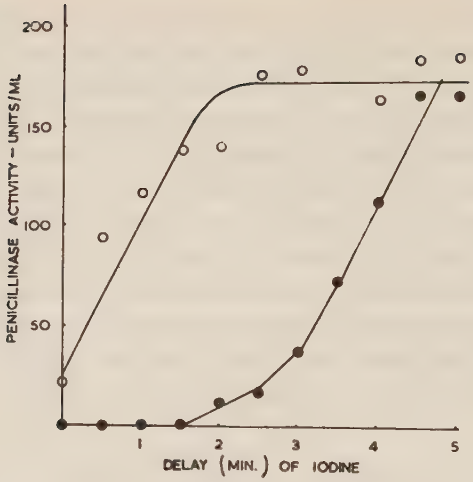

Figure 2

Survival of penicillinase activity as a function of time elapsed before addition of iodine.

(A) ○—○ expenicillinase in the γ-state

(B) ●—● CB-γ-penicillinase

be essential for the reversal to α-state[4,5]. The expression of such reversal should be, however, possible in the presence of a suitable concentration of the electrolyte, as in the case of exopenicillinase in urea (Figure 3). Accordingly the assay of the iodine-

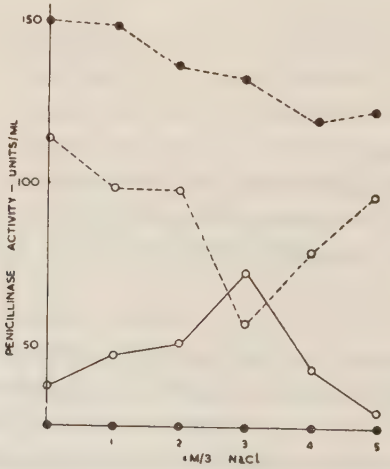

Figure 3

Effect of sodium chloride concentration on the activity of penicillinase.

○——○ *iodine-resistant* (α-type) *activity of exopenicillinase in* 3.5M *urea*

○·······○ *iodine-sensitive*(γ-type) *activity of exopenicillinase in* 3.5M *urea*

●——● *iodine-resistant* (α type) *activity of CB-γ-penicillinase*

●·····● *iodine-sensitive* (γ-type) *activity of CB-γ-penicillinase* sensitivity of CB-γ-penicillinase as a function of NaCl concentration was repeated in the presence of 3.5 M urea. The results were identical with those presented in Figure 3. It appears that the itrreversible nature of CB-γ-penicillinase is not due to an electrostatic or hydrogen-bond mediated association.

The effect of non-specific components.

The question arose whether the difference in iodine reactivity of CB-γ-penicillinase and that of exopenicillinase in the γ-state could not be accounted for (wholly or in part) by the marked difference in the degree of purity of the respective preparations. The CB-γ-penicillinase has been only partly purified and it is believed to contain a high proportion of non-penicillinase protein as well as other cell material. The possible effect of such non-specific components has been tested by repeating the above experiments with enzyme preparations deliberately contaminated with a sonic extract of non-induced cells of the adaptive penicillinase producing parent strain (*B. cereus* NRRL 569). Although the level of contamination was at least 20 times as high as that estimated for the original CB-γ-penicillinase preparation, the results obtained were identical with those reported in the previous sections.

DISCUSSION

The present observations are not directly relevant to the problems of the origin, the nature and the possible significance of the cell-bound γ-penicillinase. These have been discussed elsewhere[1,2]. It seems that we are at present justified in regarding all cell-bound penicillinase as being derived from the single penicillinase species formed by *B. cereus* and liberated as exopenicillinase. This view, first suggested by the $\alpha \rightarrow \gamma$ transition observed in exopenicillinase[2], gained further support from more recent observations. Thus there is no evidence for "intracellular" penicillinase, as all cell-bound enzyme can be located at the cell surface[7]. It has been also demonstrated that CB-γ-penicillinase does not serve as an intermediate in the formation of exopenicillinase[8]. On the other hand it has been shown that cells of *B. cereus* adsorb exopenicillinase under physiological condition. A considerable proportion of the adsorbed enzyme is indistinguishable from CB-γ-penicillinase as judged both by the serological reaction[9] and by its sensitivity to iodine[10]. The tentative definition of cell-bound γ-penicillinase as penicillinase irreversibly fixed in the γ-state[2] appears thus to be confirmed. It is not unlikely that the irreversibility of CB-γ-penicillinase is due to its being attached to fragments of the cell membrane[7]. Whatever the nature of the γ-state fixation, there is so far no indication that it is stabilized by electrostatic forces or by hydrogen bonds.

The irreversible nature of CB-γ-penicillinase may well account for its sensitivity to intermediate concentrations of iodine (cf. Figures 1 and 2). It has been shown in a previous communication[6] that urea-modified exopenicillinase readily reverts to the γ-state in the course of the enzymic reaction (Also compare curve A in Figure 2 with curve A in Figure 1). No such obvious change characterizes the behaviour of

CB-γ-penicillinase in the course of the enzymic reaction. Yet a closer examination of the data (Table I) reveals that CB-γ-penicillinase becomes definitely less sensitive to iodine as the reaction proceeds. This change does not persist, and, as soon as the substrate is exhausted or removed the enzyme is found to have fully reverted to the γ-state. It has been pointed out[9] that even in the course of the enzymic reaction CB-γ-penicillinase is not neutralized by anti-exopenicillinase serum. This observation is in agreement with the failure to establish in CB-γ-penicillinase an iodine resistant state analogous to that obtained with urea-modified exopenicillinase (cf. Figure 2). Both observations point to the fleeting nature of the change induced by the substrate in the case of CB-γ-penicillinase.

Nevertheless the evidence for the substrate-induced modification of the reactivity of CB-γ-penicillinase is essentially consistent with that obtained in the exopenicillinase-urea system[6]. In the latter case the effect of the substrate suggested a scheme to explain the apparent lack of correlation between structural changes and enzymic activity. Stated in more general terms such a scheme implies that the enzyme-substrate complex of penicillinase (whether in the α-state or in the γ-state) is essentially identical. The evidence summarized in this paper suggests that the scheme can be extended to include cell-bound γ-penicillinase.

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2-GUANIDOETHYL THIOSULPHURIC ACID

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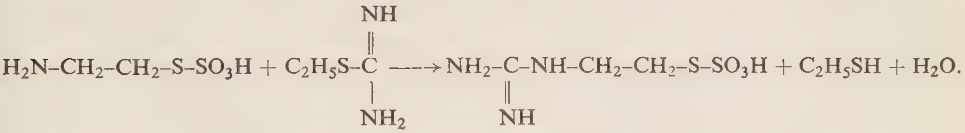
ABSTRACT

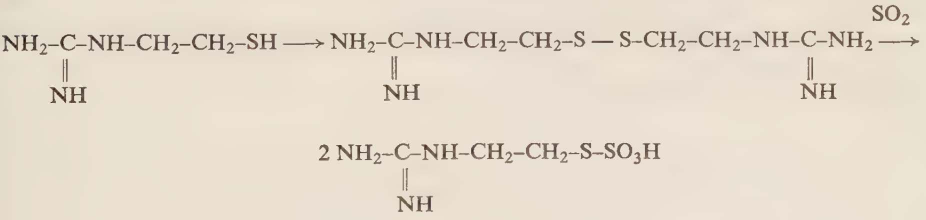
2-Guanidoethyl thiosulphuric acid was synthesized by two methods. The compound is a potential protective agent against radiation damage.

For experiments on chemical protection against ionising radiation, 2-guanidoethyl thiosulphuric acid was required. It appeared interesting because of the known protective action of 2-guanidoethyl mercaptan[1,2,3] on one hand and of aminoalkylthiosulphuric acids[4,5,6] on the other. The method employed was based on the synthesis of guanidoacetic acid[7]. 2-Aminoethylthiosulphuric acid was condensed with ethyl isothiouronium bromide in the presence of alkali.



$$H_2N{-}CH_2{-}CH_2{-}S{-}SO_3H + C_2H_5S{-}\overset{NH}{\underset{NH_2}{C}} \longrightarrow NH_2{-}\underset{NH}{C}{-}NH{-}CH_2{-}CH_2{-}S{-}SO_3H + C_2H_5SH + H_2O.$$

The same product could be prepared by an alternative method. 2-Guanidoethyl mercaptan, available from cysteamine and ethyl isothiouronium bromide, was oxidized to the corresponding disulphide and the latter treated with sulphur dioxide:



$$NH_2{-}\underset{NH}{C}{-}NH{-}CH_2{-}CH_2{-}SH \longrightarrow NH_2{-}\underset{NH}{C}{-}NH{-}CH_2{-}CH_2{-}S - S{-}CH_2{-}CH_2{-}NH{-}\underset{NH}{C}{-}NH_2 \xrightarrow{SO_2}$$

$$2\ NH_2{-}\underset{NH}{C}{-}NH{-}CH_2{-}CH_2{-}S{-}SO_3H$$

Preliminary experiments indicate that the new compound has good protective properties.

EXPERIMENTAL

Di-(2-guanidoethyl)-disulphide dihydrobromide

When 3.4 g of cysteamine and 8.2 g of ethyl isothiouronium bromide were dissolved

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in 15 ml of water, a slightly exothermic reaction took place, and ethyl mercaptan separated from the solution. The oil wdich remaned when the mercaptan and the water were removed, was oxidizedcy a current of air. The white crystals (7.8 g; 85%) were recrystallized from ethanol and melted at 192–193°.

Anal. Calcd. for $C_6H_{18}Br_2N_6S_2$: C, 18.1; H, 4.5. Found: C, 18.2; H, 4.6.

The *dipicrate* melted, after recrystallization from water, at 234–237°.

Anal. Calcd. for $C_{18}H_{22}N_{12}O_{14}S_2$: C, 31.1; H, 3.2. Found: C, 31.3; H, 3.3.

2–Guanidoethyl thiosulphuric acid

(a) A mixture of 4.0 g of the foregoing substance and 2.7 g of crystalline sodium acetate was treated with 15 ml of an aqueous (10%) solution of sulphur dioxide. After standing for some days, a white precipitate separated which was filtered, dissolved in warm water, filtered from some sulphur and allowed to crystallise. M.p. 181–183°; yield, 1.1 g (55%).

(b) When 50 ml of 2N NaOH were added to a solution of 15.7 g of 2-aminoethylthiosulphuric acid[8] and 18.5 g of ethyl isothiouronium bromide in 20 ml of cold water, ethyl mercaptan separated. After 24 hours the mercaptan and water were distilled off *in vacuo* and the crystalline residue was twice extracted with 50 ml of boiling methanol. By cooling and concentrating the solution, 4.2 g (20%) of the desired compound were obtained. M.p. and mixed m.p. with the previous sample, 181–183°.

Anal. Calcd. for $C_3H_9N_3O_3S_2$: C, 18.1; H, 4.6. Found: C, 18.5; H, 4.6.

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A COMPARATIVE STUDY OF ION-EXCHANGE EQUILIBRIA IN TRIPHOSPHATE AND COPPER TRIPHOSPHATE SOLUTIONS

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ABSTRACT

Two anion exchangers, one of low water regain and the other of high water regain, were used in order to study the sorption of triphosphate ions and copper-triphosphate complexes from the corresponding solutions. Triphosphate ion is sorbed with an average charge of 4.4 or 3.8 according to the initial ion on the exchanger (chloride or nitrate). The following complex species seem to exist under various experimental conditions: $[Cu(HP_3O_{10})]^{2-}$, $[CuCl(P_3O_{10})]^{4-}$ and $[Cu(HP_3O_{10})_2]^{6-}$.

INTRODUCTION

In a previous study[1], the sorption of triphosphate and copper triphosphate solutions on an anion exchanger of low water regain was discussed. As the sorption of triphosphate was quite low and large quantities of the original ion of the exchanger stayed on at equilibrium, it was assumed that there was some steric hindrance in the sorption of triphosphate. To check on this, a similar series of experiments were made on an exchanger of high water regain, and these results will be discussed in the present paper. Another purpose of this study was to complete and to check the experiments of the copper-triphosphate system in the presence of nitrate ions in order to compare the formation of complexes in the presence of chloride and nitrate ion and to obtain more information on the possible chloride participation in the complex as previously described. We were able to do this because an accurate modified method for the determination of nitrate has been developed by us[2,3].

EXPERIMENTAL

Procedure — ion exchange

The batch technique described by Salmon[4] was employed with 50 ml of solution and 0.500 g of anion exchanger Deacidite FF of high water regain (1.5–2.0). The solutions were agitated on an electrical shaker and it was found that four days were enough for equilibrium to be established. After separation of the aqueous and resin phases by filtration and after a quick but thorough washing of the resin, the species sorbed on it were removed by elution with 2 N hydrochloric or nitric acids (150 ml) followed by a short wash. The combined effluent and washings were diluted to 250 ml and aliquots used for analysis. Samples of the solutions in equilibrium with the resin were also analysed as a check.

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Analysis: Triphosphate was first converted to orthophosphate by boiling for thirty minutes with nitric acid and then weighed as phospho-molybdate. Copper was determined by the EDTA method with "PAN" indicator[5]. The capacity of Deacidite FF (Cl) was determined by elution with nitric acid followed by titration of the chloride ions thus released and the capacity of the nitrate form of the exchanger by elution with hydrochloric acid and titration of the nitrate ions released by the modified Leithe method[2,3]. Chloride and nitrate ions in the elutions were determined by the usual methods.

Reagents: All reagents were of analytical grade apart from the sodium triphosphate, which was purified by Quimby's method[6] and proved to be of an equivalent grade.

Resins: The anion-exchanger Deacidite FF was prepared in three different forms, chloride, nitrate and triphosphate by passing the suitable solutions through a column of the exchanger. The resins were thoroughly washed and air-dried befor use. The swelling of each resin was determined by soaking 1–2 g of resin in a small column for 2 hours, eliminating the excess of water by centrifuging and weighing the samples. Another sample of the resin was oven-dried at 110°C to constant weight so that the swelling could be expressed as percentage per dried gram of resin.

RESULTS

1. *Sorption of triphosphate ions from triphosphate solutions*

Equilibrium experiments were made with solutions of sodium triphosphate of various concentrations and Deacidite FF (w.r. 1.5–2.0) in the chloride and nitrate form. Tables I and II show these results in comparison with some of these studied previously[1] on an exchanger of low water regain (0.6–0.9).

It can be seen from Table I that while, on low water regain resin, about 20% chloride ions stayed on at equilibrium, only about 5% stayed on a high water regain resin. The average charge on the triphosphate ions sorbed is about 4.4, which is in good accordance with $pK_5 = 8.73$ and $pK_4 = 6.00$ found by Watters, Langhran and Lambert[7] and with the fact that the pH in the resin is usually much lower than in the equilibrium solution. (The calculated pH in a solution containing $HP_3O_{10}{}^{4-}$ and $P_3O_{10}{}^{5-}$ in the ratio 60 to 40 is about 8.5. This is about 0.9 pH unit lower than the average experimental pH of the equilibrium solution).

The same experiments carried out with the nitrate form of the resin show quite different results (Table II). Even on a high water regain resin, more than 20% nitrate stayed on at equilibrium and the charge on the triphosphate ions sorbed varied between 4.2 to 3.5, being in general lower than in the chloride form of the resin. It appears from these results that there is a tendency to decreasing charge with increasing concentration of triphosphate in the initial solution. This was less clear in the chloride form of the resin. In order to account for the differences in behaviour of the various forms of the exchanger (nitrate, chloride or triphosphate) their swelling was determined. The results shown in Table III were obtained.

TABLE I

Sorption of triphosphate ions by Deacidite FF-Cl

Aqueous phase			Resin phase			
Initially		Finally			Charge on	
M $Na_5P_3O_{10}$	*p*H	*p*H	N_{trip}*	N_{Cl}*	P_3O_{10} ions sorbed**	Resin
0.02	9.62	9.12	0.184	0.11	4.80	Deacidite FF-Cl w.r. 1.5–2.0
0.04	9.68	9.38	0.203	0.087	4.50	
0.08	9.62	9.42	0.205	0.107	4.30	
0.12	9.60	9.30	0.210	0.053	4.50	
0.16	9.58	9.30	0.210	0.053	4.50	
0.01	9.53	9.08	0.162	0.234	4.70	Deacidite FF-Cl w.r. 0.6–0.9
0.03	9.50	9.20	0.172	0.237	4.40	
0.04	9.58	9.39	0.153	0.340	4.30	
0.06	9.58	9.20	0.184	0.237	4.14	
0.20	9.58	9.14	0.184	0.235	4.13	

* N in this and following Tables indicates the number of moles of *x* sorbed per equivalent of resin.

** Derived from $(1\text{-}N_{Cl})/N_{trip}$.

TABLE II

Sorption of triphosphate ions by Deacidite FF-NO_3 (*w.r.* 1.5–2.0)

Aqueous phase			Resin phase		
Initially		Finally			Charge on
M $Na_5P_3O_{10}$	*p*H	*p*H	N_{NO_3}	N_{trip}	P_3O_{10} ions sorbed
0.02	9.6	9.13	0.313	0.166	4.2
0.04	9.6	9.42	0.30	0.17	4.1
0.08	9.6	9.06	0.22	0.202	3.8
0.12	9.6	9.12	0.224	0.224	3.46
0.16	9.6	9.25	0.19	0.224	3.6

TABLE III

Resin	% swelling
Deacidite FF-Cl	164.0
Deacidite FF-NO_3	132.0
Deacidite FF-P_3O_{10}	89.3

2. *Sorption of copper triphosphate complexes from solutions of copper and triphosphate*

Solutions containing various concentrations and ratios of copper and triphosphate were studied, generally in the presence of a second anion which was chloride or nitrate. These two anions were chosen as typical of groups binding (Cl^-) or not binding (NO_3^-) copper as a complex. Table IV gives these results with an anion exchanger of high and low water regain.

These results vary a great deal with the water regain of the resin. When the resin was of the low water regain type, the following results were obtained:

a) Fairly small quantities of triphosphate and copper were sorbed.

TABLE IV

Sorption of copper and triphosphate from mixed solutions of copper salts M/10 and Sodium triphosphate M/10 on Deacidite FF-X

Aqueous Phase			Resin Phase					
Initially		Finally						
Mole ratio P_3O_{10}:Cu	Other ions present X	*pH*	N_{Cu}	N_x	N_{trip}	$N_{trip\text{-}Cu}$	$\frac{N_{trip}}{N_{Cu}}$	Resin
8.0	Cl^-	7.8	0.05	0.112	0.237	0.187	4.9	w. r.
4.0	Cl^-	7.45	0.078	0.106	0.250	0.172	3.2	
2.66	Cl^-	7.08	0.112	0.133	0.259	0.147	2.3	
2.0	Cl^-	6.70	0.153	0.147	0.277	0.124	1.8	1.5 –
8.0	NO_3^-	7.78	0.04	0.26	0.195	0.155	4.9	2.0
4.0	NO_3^-	7.41	0.06	0.31	0.201	0.141	3.3	
2.66	NO_3^-	6.98	0.09	0.33	0.210	0.120	2.3	
2.0	NO_3^-	6.60	0.12	0.37	0.208	0.088	1.7	
4.4	—	8.57	0.135	—	0.280	0.145	2.07	
4.4	—	8.76	0.125	—	0.248	0.123	1.93	
2.0	NO_3^-	7.77	0.053	0.70	0.093	0.040	1.75	w.r.
2.6	NO_3^-	7.98	0.055	0.70	0.116	0.061	2.09	0.6 –
3.0	NO_3^-	8.15	0.035	0.84	0.084	0.049	2.38	
4.0	NO_3^-	8.50	0.037	0.82	0.092	0.055	2.42	0.9
1.3	Cl^-	7.29	0.237	0.595	0.239	0.020	1.01	
2.0	Cl^-	8.37	0.191	0.297	0.226	0.035	1.18	
4.0	Cl^-	8.55	0.155	0.241	0.221	0.066	1.38	

b) A very high percentage of chloride and nitrate ions stayed on at equilibrium.

c) Ratios trip:Cu were about twice as high in the presence of nitrate ions as in the presence of chloride ions. A system containing both ions showed a ratio trip:Cu of intermediate value[1]. Working on a high water regain resin, no differences were obtained in the ratios trip:Cu in the presence of nitrate or chloride ions.

In all these experiments there were always more nitrate than chloride ions left on the exchanger at equilibrium.

DISCUSSION

Comparing the sorption of triphosphate ions from triphosphate solutions by the resins of high and low water regain, it is quite clear that the cause of such large amounts of chloride ions staying on on the resin of low water regain, is steric hindrance. Nearly 95% of the available positions are occupied by triphosphate at equilibrium on the high water regain form of the exchanger. The same thing does not occur in the experiments with the exchanger in nitrate form where, even when the resin has a high water regain, 20–30% of the positions on the exchanger stay on in the nitrate form. Another difference observed on the nitrate form of the resin is the smaller charge per triphosphate ion sorbed (average 3.8 instead of 4.4 in the chloride form).

These differences mentioned here can be caused by the different swelling (Table III) of the two forms of the resin. As the swelling is less in the case of the nitrate form of the exchanger, there is a greater affinity of the resin for the nitrate ion than for the chloride ion, and this can explain the relatively large amounts of nitrate which stay on on the resin at equilibrium. The differences in the charge of the triphosphate ion sorbed can again be accounted for in terms of swelling, as the *p*H in the exchanger in the nitrate form should be lower than in the chloride form, thus possibly depressing the dissociation of the triphosphate.

Concerning the complexes formed between copper and triphosphate, we must take into account the *p*H study previously made[1], which showed that there are no complexes containing more than two triphosphate groups per ion of copper. It seems that complexes containing more triphosphate are less probable because of the large charge that such an anion complex would carry.

From the ion exchange experiments it indeed appears that there are no such complexes formed. From the experiments carried out on triphosphate solutions in the absence of copper, the anion $HP_3O_{10}^{4-}$ probably forms the complex, but the results obtained must be interpreted in terms of water regain of the resin.

On the low water regain resin in the absence of other ions, either the sorption of $[Cu(HP_3O_{10})]^{2-}$ together with free triphosphate must be taken into account, or the sorption of $[Cu(HP_3O_{10})_2]^{6-}$ without the sorption of free triphosphate. These possibilities also suit the results obtained in the presence of nitrate ions. If the complex $[Cu(HP_3O_{10})_2]^{6-}$ is formed, it is surprising that only such small quantities of copper are sorbed, in spite of the large affinity that such a highly charged complex would have.

As chloride ions are introduced into the system, the quantities of copper ions sorbed increase considerably, and the ratio trip: Cu thus decreases (Table IV). In this case, the sorption of a complex 1 trip: 1 Cu seems to be favoured. As the affinity of this complex is much higher than that formed in the presence of nitrate ions, it appears that it is of a different type. In the case of the chloride ion participating in the complex formation, the complex formed would be $[CuCl(P_3O_{10})]^{4-}$ or $[CuCl(HP_3O_{10})]^{3-}$. The higher charge of this complex would account for a higher affinity, and thus for a higher sorption than obtained in nitrate solutions. Coordination number of copper would be four, three by three oxygens (bound on three phosphorus) from the triphosphate, and the fourth by the chloride ion.

As mentioned before, there are no differences between nitrate and chloride ions on the high water regain resin. It appears from these results that the sorption of a complex 1 trip: 1 Cu is a matter of size of the complex, and therefore again caused by steric hindrance. The ratios trip: Cu are higher than in the previous experiments, and the calculations made in order to account for the capacity of the resin accord with the sorption of the complex $[Cu(HP_3O_{10})_2]^{6-}$, in which the coordination number would be six, the copper being bound to 3 oxygens of each triphosphate. It appears that this complex has a smaller affinity than the free triphosphate ion in spite of its higher charge. This must again be caused by differences in the size of the anions.

It can be concluded from this study that a solution containing an excesss of triphosphate to copper contains a mixture of the 1 trip: 1 Cu and 2 trip: 1 Cu complexes. It seems also that these two species could be separated on a suitable resin and under proper conditions.

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PAPER CHROMATOGRAPHY OF 2,4-DINITROPHENYLHYDRAZONES OF AROMATIC KETONES

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ABSTRACT

Satisfactory separation of 2,4-dinitrophenylhydrazones of alkyl aryl, cycloalkyl aryl ketones and diaryl ketones was easily effected on Whatman paper by using water-N, N-dimethylformamide as the stationary phase, and cyclohexane-carbon tetrachloride-N N-dimethylformamide (20:4:1) as the mobile phase. The *Rf* values of eight ketones and those of three typical aldehydes are given for comparison.

INTRODUCTION

For another research project[1], a reliable method was sought for detecting small amounts of both aromatic and aliphatic carbonyl compounds which had been obtained in the oxidation of certain mixtures of aromatic unsaturated hydrocarbons. Since satisfactory results were obtained by the use of paper chromatography for the separation of the 2,4-dinitrophenylhydrazones of aliphatic aldehydes and ketones[2-7] the investigation of this method was undertaken. Preliminary studies showed that the method described by Gasparis and Vecera[4] was worthy of further investigation, promising to make possible the separation and identification of aromatic ketones. This communication gives an account of paper chromatographic measurements of the *Rf* values of 2,4-dinitrophenylhydrazones of some aromatic ketones and, for comparison, some aldehydes by using water-N,N-dimethylformamide as the stationary phase, and cyclohexane-carbon tetrachloride-N,N-dimethylformamide as the mobile phase; it thus supplements similar measurements of the *Rf* values of the dinitrophenylhydrazones of aromatic aldehydes that have recently been described[8].

EXPERIMENTAL

Materials. The carbonyl compounds used in this study were obtained either from commercial sources or prepared according to the literature. The required 2,4-dinitrophenylhydrazones* were obtained by following the method described by Reich, Crane and Sanfilippo[7], and were purified by recrystallization from acetic acid, unless otherwise stated. The high purity of these compounds was evidenced latter in the course of the paper chromatographic studies. The solvents, cyclohexane (B.D.H.), N,N-

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* All melting points were determined by means of the Tottoli Melting Point Apparatus and are uncorrected.

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dimethylformamide (B.D.H.) and carbon tetrachloride ("Agan", Israel), were of analytical grade.

Procedure. Sheets of Whatman No. 1 filter paper, cut across the running direction of the fibers, were dipped into a 25%(v/v) solution of N,N-dimethylformamide in 96% ethanol and kept for 10 min, then hung in the air for 120 min. This period of time, required for the evaporation of the surplus ethanol, was found essential to obtain a clear separation between the spots.

By means of a micropipette 5μl of a solution containing 1 mg per ml of a 2,4-dinitrophenylhydrazone dissolved in xylene was placed on the starting line, 10 cm from one end of the sheet.

The prepared paper sheet was subsequently equilibrated for about 24 hours before the mobile phase was allowed to ascend. In a chamber about 40 cm long, 25 cm wide, and 55 cm high, 250 ml of the mobile phase, cyclohexane-carbon tetrachloride-N,N-dimethylformamide (20:4:1), was placed on the bottom of the chamber. The filter papers were hung on a glass rod above the mobile phase surface and allowed to equilibrate in the chamber atmosphere at 25°C for 24 hours, and then were partially immersed in the developing mixture. During a development period of 5–6 hours, the mobile phase moved about 30 cm. Subsequently, the chromatograms were dried at room temperature (25–30°C). The spots of the 2,4-dinitrophenylhydrazones were easily visible because of the colours, varying from yellow to red, typical of the derivatives of aromatic compounds. The *Rf* values measured are given in Table I.

TABLE I

Rf values and M.P.'s of 2,4-dinitrophenylhydrazones of some aromatic and aliphatic Carbonyl Compounds

2,4-Dinitrophenylhydrazone of:	M.P., °C	Reported M.P., °C	*Rf* Value
Isobutyrophenone	160	163[a]	0.90
Butyrophenone	190	190[a]	0.86
Benzophenone	232	238[a]	0.80
Propiophenone	185	190–191[a]	0.75
Cyclopropyl phenyl ketone	212	211–213[b]	0.75
Acetophenone	243	238–240[a]	0.58[c]
1-Acetylnaphthalene	248	259[d]	0.53[c]
2-Acetylnaphthalene[e]	253	261–262[f]	0.52[c]
Propionaldehyde[g]	155	155[a]	0.70
Benzaldehyde	232	237[a]	0.35
Formaldehyde[g]	166	166[a]	0.30
2,4-Dinitrophenylhydrazine	200		0

a) Huntress, E. H. and Mulliken S. p., *Identification of Pure Organic Compounds*, J. Wiley and Sons, Inc., New York, 1941. b) Mariella, R. P. and Raube, R. R., 1952, *J. Am. Chem. Soc.*, **74**, 521. c) Tendency towards "tailing". d) Williams, J. W. and Osborn, J. M., 1939, *ibid.*, **61**, 3438. e) Recrystallized from nitromethane. f) Johnson, G. D., 1953, *ibid.*, **75**, 2720. g) Recrystallized from ethanol.

RESULTS AND DISCUSSION

Since the *Rf* values vary with the quantity of the stationary phase fixed on the paper, the well reproducible figures reported here should not be taken as absolute. In testing mixtures, the same *Rf* values were obtained as for the compounds run singly. It is worthy of note, however, that larger *Rf* values were obtained when the sheets of paper, after being dipped in an ethanolic dimethylformamide solution, were dried for a period of less than two hours. Evidently, the separation between the spots in these cases was unsatisfactory. It is thus quite possible that still much better separation between members of an homologous series of aromatic ketones could be effected upon extending the above described drying period. A difference of 0.03 unit in the *Rf* values of two compounds was required to obtain a clear separation in two spots.

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ANTIMETABOLIC ACTIVITY OF AMINO ALKYL SULPHONIC ACIDS AND THEIR DERIVATIVES

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ABSTRACT
A number of α-amino alkylsulphonic acids and their peptide-like derivatives were found to cause a transient growth inhibition of *Escherichia coli* or *Proteus vulgaris*. The spontaneous reversal of the inhibition occurring at the end of the prolonged lag phase, appears to be due to the slow elaboration of metabolites, presumably amino acids accumulating in the culture medium. The growth retardation induced by the sulphonic acid analogues is nullified by the addition of an amino acid mixture to the growth medium. An hypothesis involving a feedback inhibition type of mechanism is advanced.

McIlwain prepared a number of α-amino sulphonic acids and found that these compounds inhibited bacterial growth[1].

The syntheses of a series of new amino alkyl sulphonic acids and their peptide-like derivatives have been described elsewhere[2]. The present communication reports briefly on some observations concerning the inhibitory effect of these substances on the growth of *Escherichia coli* and *Proteus vulgaris.*

The test organisms were grown in a chemically defined medium (see legend to Table I) with continuous shaking at 37°C. The compounds tested were neutralized, sterilized by Seitz filtration and added aseptically to the autoclaved medium. A standard size inoculum was used in all the experiments. Growth was measured turbidimetrically in the Klett photometer using the 55 filter.

Preliminary trials revealed that the compounds listed in Table I exhibited growth inhibitory action of a relatively low order, the minimal effective concentration ranging from 2×10^{-4}M to 10^{-3}M. As shown in Table I, the two organisms differed markedly in their susceptibility to the various analogues examined. Thus *E. coli* was inhibited mainly by the free or N-alkyl substituted α-amino alkyl sulphonic acids and only slightly, if at all, by derivatives containing a peptide or sulphonamide bond; the converse relationship was observed with *P. vulgaris.*

The suppression of growth was of a transient nature since in all instances spontaneous reversal of the inhibition took place on continued incubation. The delay in appearance of visible growth increased in a non-linear fashion with rising concentration of the inhibitor up to a limit, beyond which no further prolongation of the lag time could be

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TABLE I

Growth inhibition of E. coli and P. vulgaris by various amino sulphonic acids and their derivatives

Inhibitor	Per cent inhibition *E. coli*[a)]		*P. vulgaris*[b)]		
	15 hr	19 hr	16 hr	23 hr	23 hr
Control	0	0	0	0	0
Phenyl benzylamino methane sulphonic acid	94	70	0	0	0
Phenyl aminomethane sulphonic acid	91	70	0	0	0
α-Amino isovaleryl sulphonic acid	85	53	0	0	0
Amino methane sulphonic acid	66	28	0	0	0
α-Amino heptane sulphonic acid	38	7	0	0	0
Phthalyl tauryl glycyl glycine ethyl ester	0	0	60	41	0
Anisyl benzylamino methane sulphonic acid	0	0	50	32	0
Phthalyl tauryl glycine ethyl ester	0	0	40	26	0
Hippuryl amino methane sulphonic acid	0	0		83	35
Acetyl amino methane sulphonic acid	0	0		80	30
Carbobenzoxy amino methane sulphonic acid	0	0		70	17
Carbobenzoxy glycyl amino methane sulphonic acid	0	0		57	7

a) The compositon of the culture medium was: $Na_2HPO_4 \cdot 12H_20$, 0.8%; KH_2PO_4, 0.7%; $MgSO_4 \cdot 7H_2O$, 0.02%; $(NH_4)_2SO_4$, 0.2%; $CaCl_2$, 0.0005%; D-glucose, 0.25%; inhibitor, 10^{-3}M.

b) Culture medium as above, but supplemented with 0.01% niacin and 0.0025% tryptophan; concentration of inhibitor, 5×10^{-4}M.

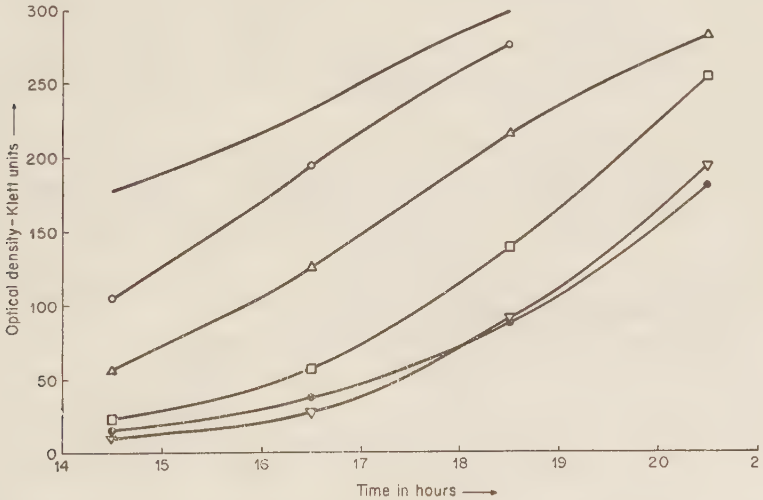

Figure 1

Rate of growth of E. coli in the presence of amino alkyl sulphonic acid and their derivatives

—— Control
○——○ α- Amino heptane sulphonic acid
△——△ Amino methane sulphonic acid
□——□ α- Amino isovaleryl sulphonic acid
●——● Phenyl amino methane sulphonic acid
▽——▽ Phenyl benzylamino methane sulphonic acid;
Experimental conditions as for Table I.

demonstrated. The density of the bacterial cultures ultimately attained was the same as in the control, regardless of the antimetabolite concentration employed. Furthermore, even at the highest concentration level of the inhibitor, once the logarithmic phase of proliferation had been reached, the rate of cell multiplication was essentially equal to that in the control culture (see Figure 1). It appears therefore that the growth inhibition induced by the amino alkyl sulphonic acids bears primarily on the lag phase. It may be mentioned that a similar type of antagonistic action has been observed with other antimetabolites[3].

The described inhibition pattern suggested that the onset of growth, following the prolonged lag period might be due to the slow formation and accumulation in the bacterial cells of a metabolite capable of reversing the inhibitory effect of the amino sulfonic acid. The validity of this interpretation appears to be borne out by the finding that 24 hours' cultures of *E. coli*, when sterilized by Seitz filtration and added in a proportion of 1:10 to the fresh culture medium, completely annulled the inhibitory action of the amino sulphonic acids tested.

The growth retarding action of the amino alkyl sulphonic acids on *E. coli* could be readily overcome by a mixture of natural amino acids, thus attesting to the antagonistic relationship prevailing between the two groups of compounds. Furthermore, the inhibitory effect exerted by α-amino isovaleryl sulphonic acid (10^{-3}M), a compound selected for a more detailed investigation of the mode of action, was nullified by L-valine (5×10^{-5}M) but not by L-leucine nor by L-isoleucine.

The mechanism mediating the antimetabolic activity of the amino alkyl sulfonic acids is not yet clear. An attractive hypothesis which is open to experimental trial can be formulated as follows:

The amino sulphonic acid analogues act by inducing a feedback inhibition[4] of the biosynthesis of the corresponding amino carboxylic acids, though unable to fulfil the specific functions of the latter compounds in the cell metabolism.

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RESOLUTION OF N-BENZYL-DL-ASPARTIC ACID

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ABSTRACT
N-Benzyl-DL-aspartic acid has been resolved by means of either ethyl L-leucinate or L-leucine amide. With ethyl L-leucinate pure N-benzyl-L-aspartic acid is obtained, in contradistinction to L-leucine amide which separates the diastereoisomer containing N-benzyl-D-aspartic acid.

α- and β-DL-aspartyl peptides and amides have been prepared from N-benzyl-DL-aspartic acid by either employing a mixed anhydride with chlorocarbonic acid[1,2,3], or via N-benzyl-DL-aspartic anhydride hydrochloride[4]. Since these methods do not suffer from the disadvantage of difficult separation of α- and β-isomers, experienced when working with N-benzyloxycarbonyl-L-aspartic anhydride[5], it was thought desirable to resolve N-benzyl-DL-aspartic acid with a view to utilizing its enantiomorphs for the preparation of optically pure α- and β-aspartyl amides and peptides.

Trials to use alkaloids, such as brucine, cinchonine, morphine, etc., as resolving agents were unsuccessful, but when turning to esters of natural amino acids, it was ound that especially ethyl L-leucinate was well suited and complete separation of the -isomer could be effected by several recrystallizations of the diastereoisomeric salts rom water. N-Benzyl-L-aspartic acid was then obtained by dissolving the salt in lkali, extracting the L-leucine ester with ether and acidification with hydrochloric cid to *p*H 3. Hydrogenolysis by means of palladium chloride on charcoal yielded -aspartic acid which gave the same optical rotation as an authentic sample. Isolation f the D-isomer, however, from the mother liquor proved difficult. By elimination f the bulk of the L-L-salt through repeated recrystallization from water and subsequent reatment with alkali and hydrochloric acid, as above, N-benzyl-D-aspartic acid was btained, which, according to its optical rotation, was of about 75% purity.

The fact that the L-leucine ester (or most other α-amino acid esters at that) is very abile and could hardly be recovered after use, being converted into the diketopiperaine, prompted us to try L-leucine amide as resolving agent. This could be prepared n good yields either from the free ethyl ester or, alternatively, from its hydrochloride, y treatment with a saturated solution of ammonia in dry methanol[6]. The diastereisomeric salts could be separated by crystallization from ethanol and this time the -leucine amide salt with N-benzyl-D-aspartic acid proved to be the less soluble, hereby affording the complete separation of N-benzyl-D-aspartic acid. A smaller uantity of N-benzyl-L-aspartic acid could also be isolated by two more recrystal-

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lizations of the second diastereoisomer, i.e., the salt of L-leucine amide and N-benzyl-L-aspartic acid, which precipitated from the original mother liquor after the separation of L-leucine amide — N-benzyl-D-aspartic acid. The respective N-benzyl-aspartic acids were obtained by acidification with hydrochloric acid to *p*H 3–4 of an aqueous solution of the salt. L-Leucine amide could then be recovered by adding alkali, evaporating the water in vacuo and extraction with hot chloroform in which only L-leucine amide is soluble.

EXPERIMENTAL

*Salt of N-benzyl-*L*-aspartic acid with ethyl* L*-leucinate.*— N-Benzyl-DL-aspartic acid[7] (22.3 g) was suspended in water (20 ml) and ethyl L-leucinate[8] (15.9 g) added. On warming on a water bath, a clear solution was obtained. This was left undisturbed overnight and the salt of N-benzyl-L-aspartic acid with ethyl L-leucinate precipitated meanwhile. (If this is not the case, crystallization may be induced by shaking). After filtration, the salt was recrystallized from 8 ml of water. Average yield was 10 g (51 %); m.p. 190° (sintering commences at about 90°); $[\alpha]_D^{24} = -19.2°$ ($c = 0.125$ in 5 % sodium bicarbonate solution).

Anal. Calcd. for $C_{19}H_{30}O_6N_2 + 1/2\ H_2O$: C, 58.2; H, 7.9; N, 7.1. Found: C, 58,0; H, 7.6; N, 6.9.

*N-Benzyl-*L*-aspartic acid.*— The above salt (6.5 g) was dissolved in a 10 % sodium hydroxide solution (7 ml). After several extractions with ether, the aqueous solution was acidified with hydrochloric acid to *p*H 3. N-Benzyl-L-aspartic acid was obtained as white crystals; yield 2.9 g (80 %); m.p. 209°; $[\alpha]_D^{24} = -33.4°$ ($c = 0.12$ in 5 % sodium bicarbonate solution).

Anal. Calcd. for $C_{11}H_{13}O_4N$: N, 6.3. Found: N, 6.2.

L-Aspartic acid. N-Benzyl-L-aspartic acid (1.6 g) was suspended in glacial acetic acid (35 ml) and 0.2 g of a palladium chloride on carbon (Norit) catalyst (30 %) added. The reduction was carried out in a Parr low pressure apparatus for 5 hours. The cold reaction mixture was filtered. The bulk of the substance which adhered to the catalyst could be extracted with cold formic acid and precipitated with acetone. This as well as the part recovered by evaporation of the acetic acid yielded 0.8 g (84 %); $[\alpha]_D^{26} = +23°$ ($c = 0.1335$ in 1 N hydrochloric acid). The optical rotation was the same as that of an authentic sample of L-aspartic acid (from N.B. Co.).

Anal. Calcd. for $C_4H_7O_4N$: N (Kjeldahl), 10.5; N (Van Slyke), 10.5. Found: N (Kjeldahl), 10.6; N (Van Slyke), 10.7.

*Salt of N-benzyl-*D*-aspartic acid with ethyl* L*-leucinate.*— After separation of the salt of N-benzyl-L-aspartic acid with ethyl L-leucinate, a second crop of crystals was obtained from the original mother liquor, which did not give an appreciable rotation. A third crop, however, after recrystallization from water, gave 1.5 g. of a salt which was dextrarotatory. $[\alpha]_D^{27} = +9.1°$ ($c = 0.143$ in 5 % sodium bicarbonate solution).

*N-Benzyl-*D*-aspartic acid.* Treatment of the salt with alkali and hydrochloric acid as above, gave N-benzyl-D-aspartic acid; m.p. 203°; $[\alpha]_D^{24} = +24°$ ($c = 0.12$ in 5% sodium bicarbonate solution). This corresponds to a resolution of about 75%.

L-*Leucine amide.*

(A) From ethyl L-leucinate.

This substance was prepared according to the instructions given for DL-leucine amide[6]. The yield was about 90%; m.p. (after recrystallization from benzene) 99–100°; $[\alpha]_D^{23} = +6°$ ($c = 0.25$ in water).

Anal. Calcd. for $C_6H_{14}N_2O$: C, 55.2; H, 10.8; N, 21.6. Found: C, 55.3; H, 10.6; N, 21.5.

(B) From ethyl L-leucinate hydrochloride.

The ester hydrochloride (25 g) was dissolved in dry methanol (300 ml) and dry gaseous ammonia was passed into the solution until saturation. The reaction mixture was shaken at room temperature for four days. The *p*H was then adjusted to 10 with sodium hydroxide solution and evaporation to dryness *in vacuo* was carried out. The amide was extracted with hot chloroform and after evaporation of the solvent the substance was recrystallized from benzene. Yield 9 g (54%).

*Salt of N-benzyl-*D*-aspartic acid with* L*-leucine amide.*— N-Benzyl-DL-aspartic acid (5.5 g) suspended in ethanol (60 ml) was heated on a water bath with L-leucine amide (3.5 g) until a clear solution resulted. After a short time the salt crystallized in needles united to bundles. After filtration the substance was recrystallized from ethanol, yielding 2.7 g (60%); m.p. 180–182° (sintering commences at about 115°); $[\alpha]_D^{23} = +26°$ ($c = 0.125$ in water).

Anal. Calcd. for $C_{17}H_{27}O_5N_3$: C, 57.6; H, 7.7; N, 11.9. Found: C, 57.2; H, 8.2; N, 11.6.

*N-Benzyl-*D*-aspartic acid.* The above salt (2 g) was dissolved in water (2 ml) and acidified with hydrochloric acid to *p*H 4. N-Benzyl-D-aspartic acid precipitated. After addition of ethanol (5 ml), the substance was filtered and dried. Yield 1.24g (98%); m.p. 209°; $[\alpha]_D^{23} = +33.3°$ ($c = 0.125$ in 5% sodium bicarbonate solution).

*Salt of N-benzyl-*L*-aspartic acid and* L*-leucine amide.* After separation of the salt of N-benzyl-D-aspartic acid with L-leucine amide, the second diastereoisomeric salt crystallized. This on recrystallization from ethanol yielded 1.5 g (33.5%); $[\alpha]_D^{25} = -14°$ ($c = 0.125$ in water).

*N-Benzyl-*L*-aspartic acid.* Liberation from the salt as for the D-isomer. $[\alpha]_D^{23} = -33.2°$ ($c = 0.125$ in 5% sodium bicarbonate solution); m.p. 209°.

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THE XXVIth SCIENTIFIC MEETING
ISRAEL CHEMICAL SOCIETY
SECTION OF ORGANIC CHEMISTRY
AND
BIOCHEMISTRY

Jerusalem, April 19—20, 1960

SECTION A

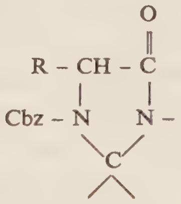
ORGANIC CHEMISTRY

First Session Chairman: D. Lavie

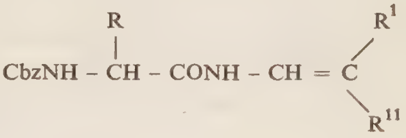
The reactions of carbobenzoxyamino acid amides with carbonyl compounds

Uri Zehavi and Dov Ben-Ishai*, *Technion–Israel Institute of Technology, Haifa*

Carbobenzoxyamino acid amides have been found to react with carbonyl compounds in the presence of a sulphonic acid catalyst to give two main types of products (I, II). Type I comprises derivatives of 4-imidazolidones while type II has the ene-amide structure. The reaction products are predetermined by the structures of both the amide and the corresponding carbonyl component.



(I)



(II)

Catalytic hydrogenation converts both types of products into compounds whose structure was proved by comparison with authentic samples.

In order to investigate the possibility that the incorporation of an amide bond into an imidazolidone ring might change its chemical properties, the acid hydrolysis of the imidazolidones was also studied.

Synthesis of α-amino-β-hydroxy acids by reaction of 2,3-epoxy-acids with benzylamine

Y. Liwschitz* and Y. Rabinsohn, *Department of Organic Chemistry, The Hebrew University of Jerusalem*

Epoxy acids derived from α, β-unsaturated fatty acids, can be transformed into α-amino-β-hydroxy acids by reaction with benzylamine. The epoxidation is efficiently performed by hydrogen peroxide in the presence of sodium tungstate[1]. The opening of the oxirane ring by benzylamine is a stereospecific reaction, producing only one diastereoisomeric pair. Thus, only N-benzyl-DL-allothreonine is formed from 2,3-epoxybutyric acid and neither N-benzyl-DL-threonine nor the two N-benzyl-*α*-hydroxy-*β*-amino-butyric acids have been found in the reaction mixture. It may, therefore, be assumed that in the reaction of benzylamine with cis-epoxysuccinic acid and epoxy-methylsuccinic acid, derived from maleic and citraconic acid respectively, N-benzyl-*β*-hydroxy-DL-aspartic acid and N-benzyl-*β*-methyl-*β*-hydroxy-DL-aspartic acid of erythro configuration are the sole products, which could be isolated in good yields. Removal of the N-benzyl group by catalytic hydrogenation with palladium chloride on charcoal gave the free amino acids. The *β*-hydroxy-DL-aspartic acid obtained by us, seems to be identical with the so-called "para" isomer prepared by Dakin[2] from *β*-chloromalic acid, for which no configurational assignment has been published so far. Greenstein and co-workers[3], who prepared *β*-methyl-*β*-hydroxy-DL-aspartic acid by an entirely different route, obtained a mixture of the diastereoisomers, the components of which were not characterised.

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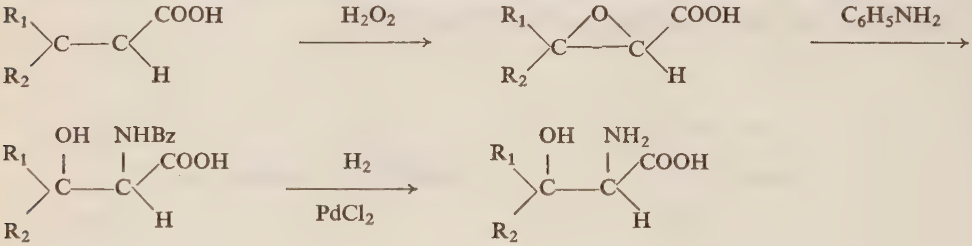
* Lecturer



allothreonine: $R_1 = CH_3$ $R_2 = H$

β-hydroxyaspartic acid: $R_1 = COOH$ $R_2 = H$

β-methyl-*β*-hydroxy-aspartic acid: $R_1 = COOH$ $R_2 = CH_3$

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Synthesis of *α*-dibenzylamino-*γ*-butyrolactone, and of protected homoserine and *α*,*γ*-diaminobutyric acid derivatives

Max Frankel, Y. Knobler and T. Sheradsky, *Department of Organic Chemistry, The Hebrew University of Jerusalem*

α-Dibenzylamino-*γ*-butyrolactone (I) was prepared from *α*-bromo-*γ*-butyrolactone[1] and dibenzylamine as the result of *α*-amination only. The lactone form of homoserine, the α-amino group being masked by two benzyl groups, was used in the synthesis of protected α-amino acids which differ in their γ-substituents according to the manner of ring-opening. By controlling the degree of debenzylation, and by varying the medium, the same starting material could also be used in the preparation of homoserine, of its derivatives, and of its lactonic and dimeric forms. The latter compounds are also intermediates in the synthesis of other natural amino acids[2].

Catalytic hydrogenation of I, with the aid of palladium black in ethanol containing hydrochloric acid, gave α-monobenzylamino-γ-butyrolactone hydrochloride (II). When a palladium chloride catalyst was used in the presence of hydrochloric acid, both benzyl groups were removed, giving α-amino-γ- butyrolactone hydrochloride (III). With palladium on charcoal, hydrogenation in a neutral medium gave free α-amino-γ-butyrolactone, which dimerised to the diketopiperazine (IV) of homoserine on further heating. Catalytic hydrogenation of I, using palladium on charcoal in aqueous ethanolic solution in the presence of triethylamine (as a basic reagent for the prevention of relactonisation), gave α-amino-γ-hydroxybutyric acid (homoserine) (IVa).

Reaction between I and benzylamine gave α-dibenzylamino-γ-hydroxy-N-benzyl-butyramide (V), by irreversible opening of the lactone ring. α-Monobenzylamino-γ-hydroxy-N-benzyl-butyramide (VI) was prepared directly from α-bromo-γ-butyrolactone and benzylamine, with simultaneous α-amination and ring opening to the amide. On ring opening of I with alkali, followed by acidification in the cold, N,N-dibenzyl-homoserine (VII) was isolated. Ring closure back to I could be achieved by heating, a reaction which proceeded faster in acidic medium.

Opening of I with potassium phthalimide in dimethylformamide (150°) resulted in a masked α,γ-diaminobutyric acid (IX), whose amino groups are selectively protected, by two benzyl groups on the α-nitrogen, and by the phthalyl group on the γ-nitrogen. Hydrolytic removal of the phthalyl group with hydrobromic acid gave α-dibenzylamino-γ-aminobutyric acid hydrobromide (X); the latter was freed by triethylamine in ethanol to give α-dibenzylamino-γ-aminobutyric acid (XI).

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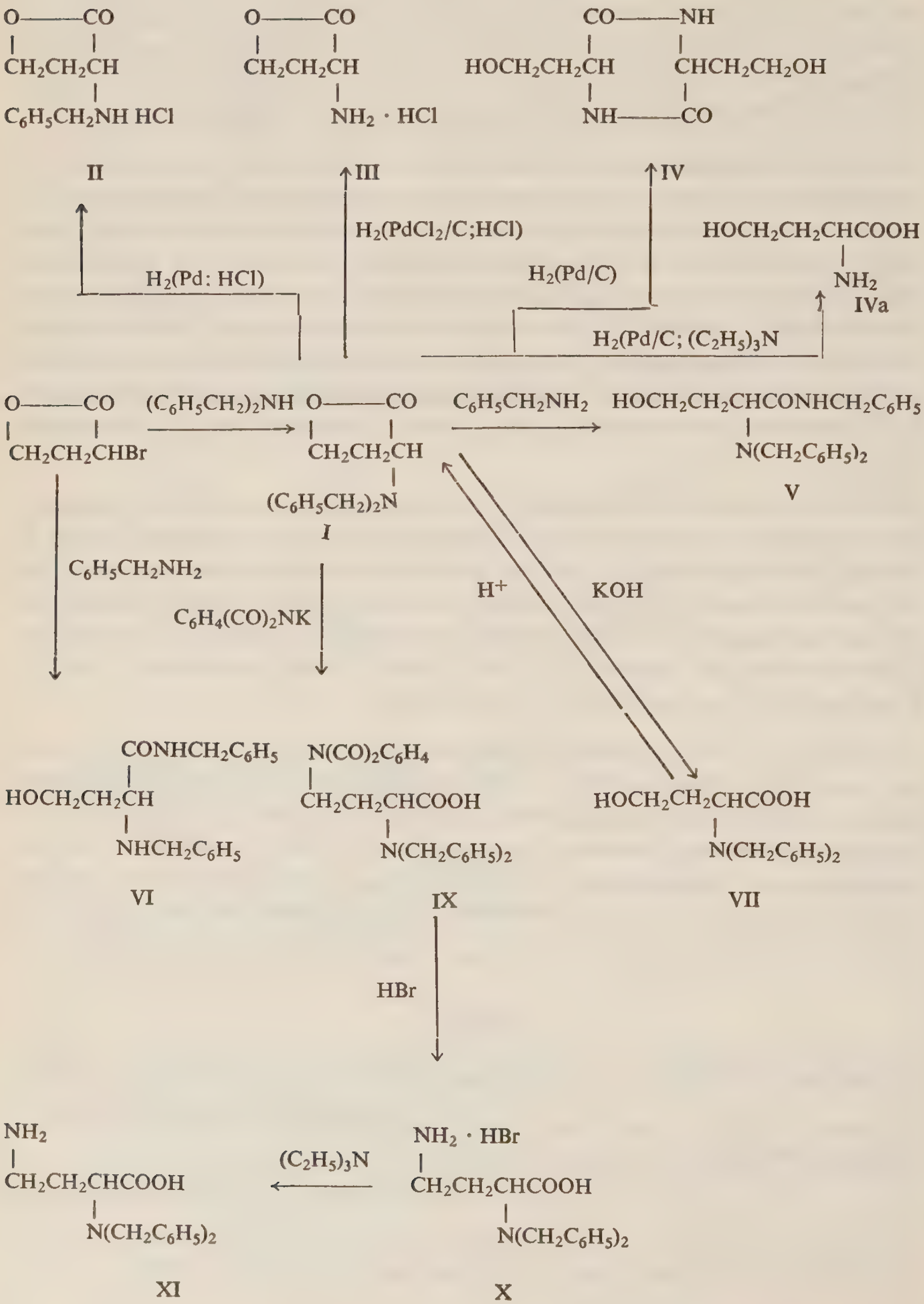
TABLE


II
III
IV
IVa
I
V
VI
IX
VII
X
XI
H2(Pd: HCl)
H2(PdCl2/C;HCl)
H2(Pd/C)
H2(Pd/C; (C2H5)3N
(C6H5CH2)2NH
C6H5CH2NH2
C6H5CH2NH2
C6H4(CO)2NK
H+
KOH
HBr
(C2H5)3N
HOCH2CH2CHCOOH NH2
HOCH2CH2CHCONHCH2C6H5 N(CH2C6H5)2
CONHCH2C6H5 HOCH2CH2CH NHCH2C6H5
N(CO)2C6H4 CH2CH2CHCOOH N(CH2C6H5)2
HOCH2CH2CHCOOH N(CH2C6H5)2
NH2 · HBr CH2CH2CHCOOH N(CH2C6H5)2
NH2 CH2CH2CHCOOH N(CH2C6H5)2

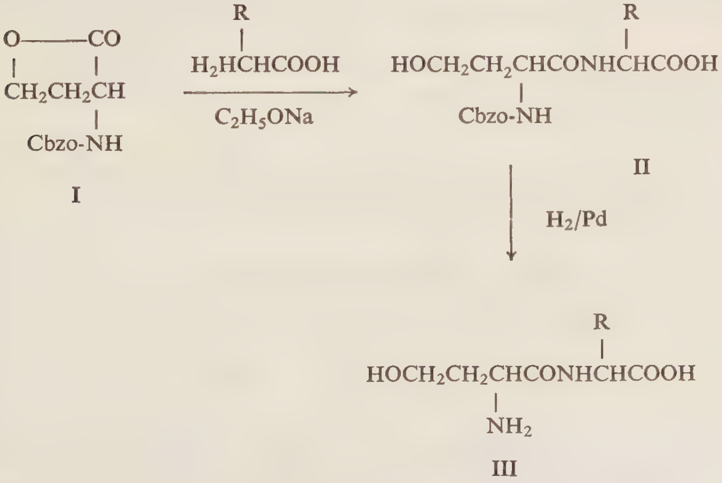
Synthesis of peptides of homoserine and of α,γ-diaminobutyric acid by an aminolactone method

MAX FRANKEL, Y. KNOBLER AND T. SHERADSKY, *Department of Organic Chemistry, The Hebrew University of Jerusalem*

The preparation of peptides and polypeptides of homoserine (α-amino-γ-hydroxybutyric acid) is complicated by the fact of the lactonisation of the acid and its reactive derivatives. In the present work, the irreversible transition from the internal-ester form to the open amide was exploited in the synthesis of peptides of homoserine. An important advantage of the method presented here is the fact that these peptides are prepared using an intermediate stage of the homoserine synthesis, thus omitting the preparation of the amino acid itself, and of the subsequent protected intermediate, needed in the peptide synthesis.

α-Carbobenzoxyamino-γ-butyrolactone[1] (I), the carbobenzoxy derivative of the lactonic form of homoserine, was used as the starting material, and reacted with amino acid salts in ethanolic solution. N-Carbobenzoxyhomoseryl peptides (II) were obtained and hydrogenated to the free homoseryl peptides (III). By this method the following new peptides have already been prepared: homoseryl-alanine, homoseryl-β-alanine, homoseryl-phenylalanine, homoseryl-leucine and homoseryl-serine.

TABLE I

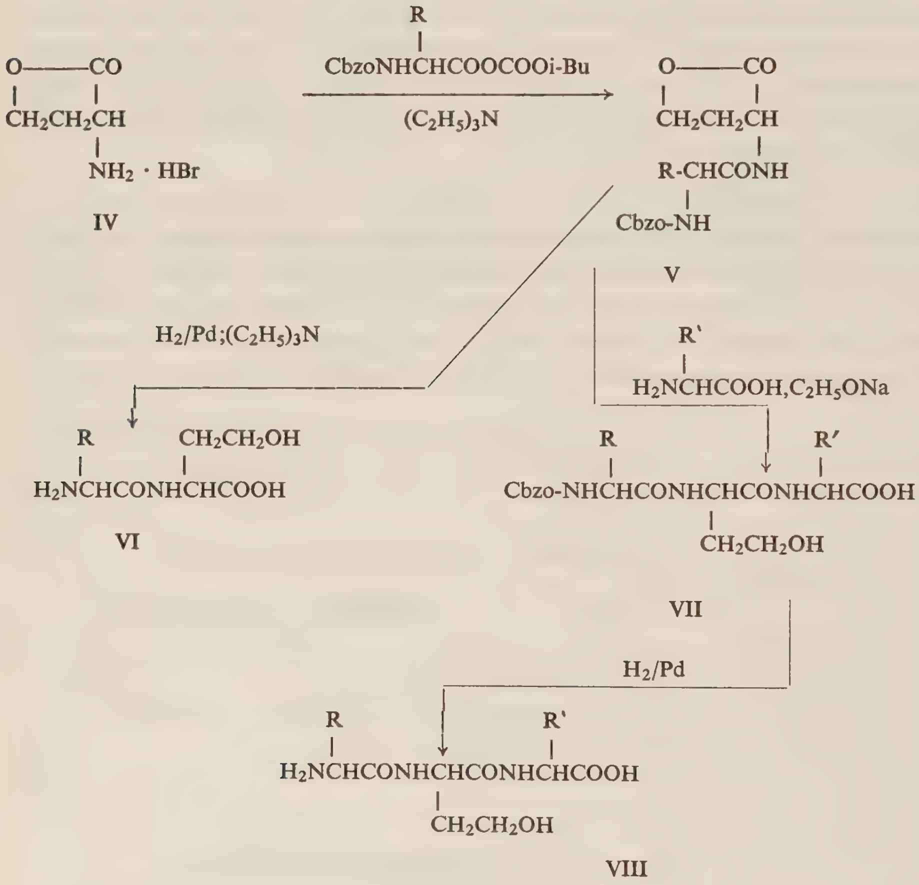


$$\underset{\text{I}}{\underset{\text{Cbzo-NH}}{\overset{\text{O——CO}}{CH_2CH_2CH}}} \xrightarrow[C_2H_5ONa]{\overset{R}{H_2HCHCOOH}} \underset{\text{II}}{HOCH_2CH_2\underset{\text{Cbzo-NH}}{CH}CONH\overset{R}{CH}COOH} \xrightarrow{H_2/Pd} \underset{\text{III}}{HOCH_2CH_2\underset{NH_2}{CH}CONH\overset{R}{CH}COOH}$$

By the action of carbobenzoxyamino acids on α-amino-γ-butyrolactone hydrobromide (IV), using the mixed anhydride method, carbobenzoxypeptido-lactone (V) was prepared, and from this the free N-homoserine peptide (VI) was obtained by catalytic reduction in a basic medium (triethylamine). The carbobenzoxy tripeptide

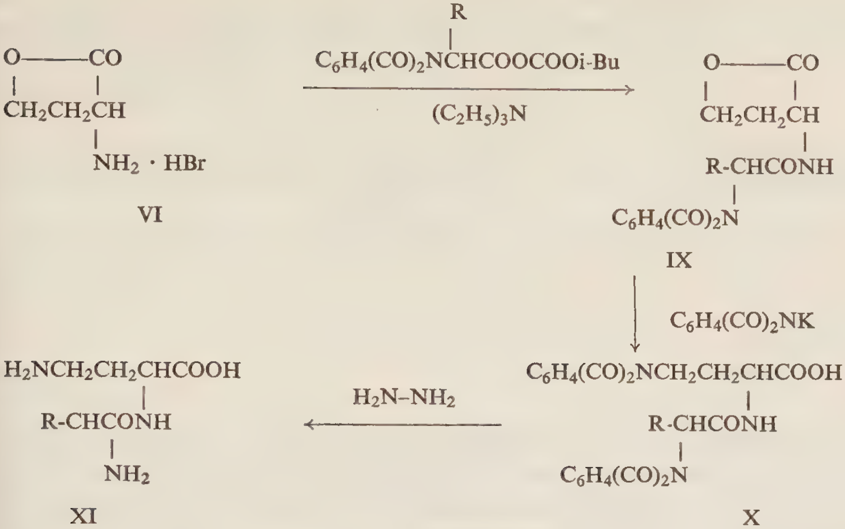
(VII) was prepared by the reaction between carbobenzoxy peptido-lactone (V) and an amino acid salt. Catalytic hydrogenation of VII gave the free tripeptide (VIII), of which the homoserine unit is the middle unit. In this way the construction of a controlled chain, in which homoserine alternates with other amino acids, may be continued.

TABLE II



By the action of a phthalylamino acid on α-amino-γ-butyrolactone hydrobromide (IV), using the mixed anhydride method, phthalylpeptidolactone (IX) was synthesized. Opening of this lactone by potassium phthalimide, with γ-introduction of the phthalimide group, gave an α,γ-diamino-butyric acid peptide, connected by a peptide linkage through the α-nitrogen with a phthalylamino acid, while the γ-nitrogen is protected by the phthalyl group (X). Removal of the two phthalyl groups with the aid of hydrazine leads to the α-peptide (XI) of α,γ-diaminobutyric acid.

TABLE III



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The bromination of indoles in aqueous and nonaqueous media[1]

Abraham Patchornik[2], Wiliam B. Lawson and Bernhard witkop, *National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md., U.S.A.*

The moderate yields of cleavage at the C-tryptophyl pepeptide bond when tryptophan peptides were treated with N-bromosuccinimide indicated the occurrence of side reactions.

Bromination of indoles in aqueous and nonaqueous media may follow two pathways. Thus 2:6 dibromoskatole (I) resulted from the action of N-bromophthalimide on skatole in benzene. The structure of this compound was proved by acid hydrolysis to yield 6–bromo-3 methyl oxindole (II) and by oxidative degradation with ozone to the 2-acetamino-4-bromobenzoic acid (III).

5-Bromo-3-methyl oxindole (IV), the isomer of II, was obtained by treatment of skatole with N-bromosuccinimide in dilute acetic acid solution.

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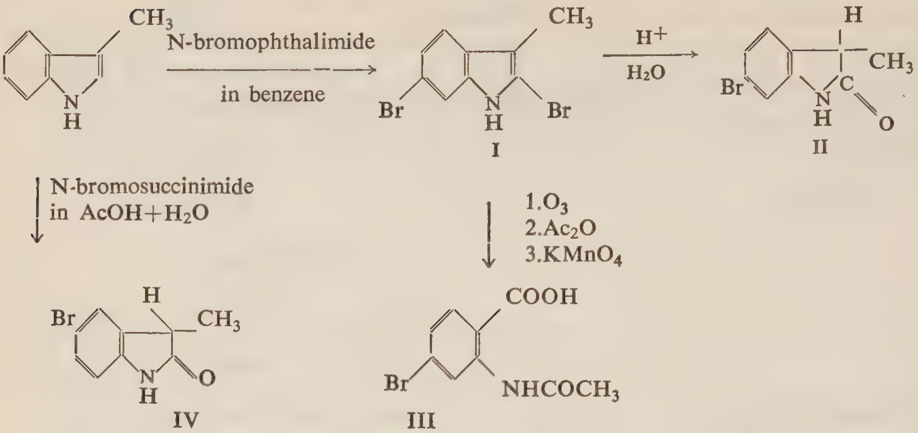
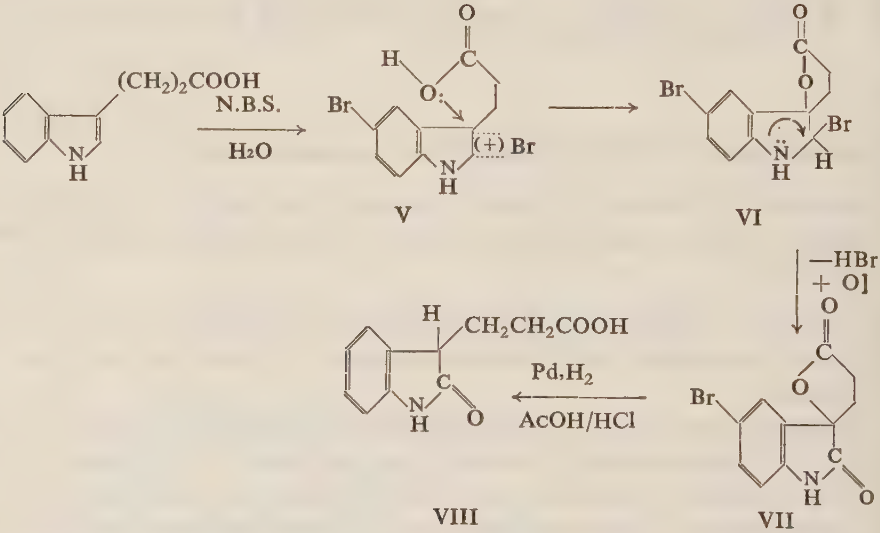
[1] Research carried out at The National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, U.S. Department of Health, Education and Welfare.

[2] On leave of absence from the Weizmann Institute of Science.



In aqueous media (5-bromo) dioxindolespirolactone (VII) was obtained. This, when hydrogenated with Pd/C, yielded oxindole-3-propionic acid (VIII). It is possible that this lactone is formed by intramolecular action of the carboxyl group on a bromonium intermediate (V).



Preparation and decarboxylation of α-cyanocinnamic acids

JOSEPH KLEIN AND A. MEIR*, *Department of Organic Chemistry, The Hebrew University of Jerusalem*

Substituted α-cyanocinnamic acids were prepared from the acetals of substituted benzaldehydes and canoacetic acid without added catalyst, i.e. under conditions under which the free aldehydes do not enter any reaction. The yields obtained by this method are often higher than those secured in the usual alkali-catalysed condensation between the free aldehydes and cyanoacetic acid.

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* Lecturer

Decarboxylation of these acids in pyridine solution leads to equilibrium mixtures of the substituted *cis*- and *trans*-cinnamonitriles, in which the *trans*-forms prevail.

The thermal and the quinoline-induced decarboxylations give mostly the less stable isomer.

Second Session Chairman: D. BEN-ISHAI

A phosphorylation product of poly-DL-serine

YEHUDA LAPIDOT AND EPHRAIM KATCHALSKI, *Department of Biological Chemistry, The Hebrew University of Jerusalem*

Phosphorus–containing proteins such as phosphvitin and casein are abundant in nature. Most of the phosphoric acid residues are bound in these proteins by means of an ester link to the hydroxyl group of serine[1,2]. Since this phospho-ester link is considerably more labile than the corresponding link in phosphoserine, it was interesting to synthesize poly-*o*-phosphoserine and to study its chemical behavior.

A sixty percent phosphorylation of poly-DL-serine[3] could be accomplished by means of chlorophosphoric acid[4]. A mixture of poly-DL-serine and chlorophosphoric acid was maintained at 40° for 24 hours, and the phosphorylation product precipitated from the clear solution obtained with alcohol-ether. The phosphorylation product of poly-DL-serine thus obtained (I) is readily soluble in water and is insoluble in alcohol or ether. It contains 15.5% organically-bound phosphorus and 10.4% nitrogen (Kjeldahl) by weight.

The presence of orthophosphate bonds and the absence of pyro- or polyphosphate bonds in (I) could be demonstrated as follows:

(a) On incubation with acid phosphatase all the phosphate of (I) appeared as inorganic phosphate[5]. (b) On heating with 1N HCl for seven minutes, (I) liberates a negligible amount of inorganic phosphate[6].

The orthophospho-bonds of polyphospho-DL-serine (I) proved to be considerably more labile towards acid phosphatase, acid (boiling in 1N HCl) and alkali (0.5N NaOH at 37°), than that of *o*-phospho-DL-serine[6].

Work on the synthesis of polyphospho-L-serine is now underway, and its behavior towards enzymes, acids and bases will be studied.

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* Neuhaus used the chlorophosphoric acid for preparing *o*-phospho-DL-serine.

Monomeric derivatives and polymers of S-acetyl cysteine

Tsvi Sadeh, *Department of Biophysics, The Weizmann Institute of Science, Rehovot*

S-acetyl-L-cysteine hydrochloride (I) was obtained by the acetylation of L-cysteine hydrochloride with acetyl chloride in a mixture of acetic and hydrochloric acids[1], m.p. 135°; $[\alpha]_D^{24}$–48.5°, in water. (I) yields N-carboxy-S-acetyl-L-cysteine anhydride (II) on phosgenation in dioxane, m.p. 67°. The anhydride (II) was polymerized in dioxane to give poly-S-acetyl-L-cysteine (III) with an average molecular weight of 18,000 (sedimentation and diffusion). Rapid deacetylation of (III) occurs in dilute aqueous ammonia. The poly-L-cysteine thus formed was identical in its properties with that synthesized by Berger *et al*[2].

Copolymers containing S-acetyl-L-cysteine and B-benzyl-L-aspartate residues in a molar ratio of 1:1 and 2:1 were obtained by the copolymerization of the corresponding N-carboxyanhydrides. On treatment of III with anhydrous hydrogen bromide in glacial acetic acid a copoly-S-acetyl-L-cysteine-L-aspartic acid (IV) was obtained. Copolymer (IV), on alkaline hydrolysis, was rapidly deacetylated, as to be expected.

S-acetyl-N-carbobenzoxy-DL-cysteine (V) was obtained by the reduction of N′, N′-dicarbobenzoxy L-cysteine[4] with zinc in acetic acid, followed by acetylation with acetyl bromide and pyridine; m.p. 117–118°. The acetylation process adopted led to complete racemization. The racemic compound (V) was also obtained by the addition of thioacetic acid to N-carbobenzoxy dehydroalanine; (V) could obviously not be used in the synthesis of peptides containing optically active cysteine residues.

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Poly-DL-homocysteine, preparation and properties

TSVI SADEH AND EPHRAIM KATCHALSKI, *Department of Biophysics, Weizmann Institute of Science, Rehovot*

The synthesis of poly-DL-homocysteine was carried out as follows:
N-carboxy-S-benzyl-DL-homocysteine anhydride (I, m.p. 33°) was obtained by phosgenation of S-benzyl-DL-homocysteine[1] in dioxane. On polymerization in dioxane, using triethylamine as initiator, (I) yielded poly-S-benzyl-DL-homocysteine(II) with an average molecular weight of 15,000 (as determined viscometrically in dichloroacetic acid). Poly-DL-homocysteine (III) was derived from (II) upon reduction with sodium in liquid ammonia. It is of interest to note that poly-S-benzyl-L-cysteine could not be reduced under similar conditions[2]. Poly-DL-homocysteine (III) resembles in its chemical properties poly-L-cysteine[2]. It reacts quantitatively with iodoacetic acid as well as with chloromercuribenzoate. (III) dissolves in dilute alkali at pH values above 8. On oxidation with performic acid it yields poly-DL-homocysteic acid. The latter resembles poly-L-cysteic acid in its electrochemical behaviour.

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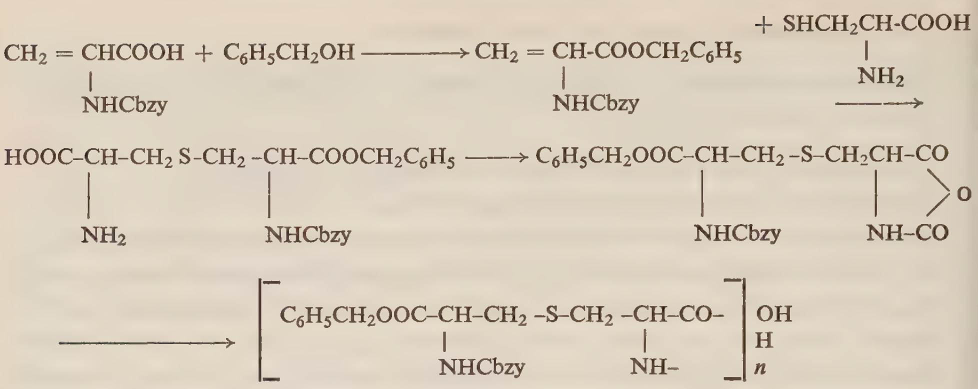
Syntheses of some polymeric polypeptides of lanthionine

MAX FRANKEL AND DAVID GERTNER, *Department of Organic Chemistry, The Hebrew University of Jerusalem*

Lanthionine, a tetrafunctional dicarboxy diamino acid, containing a sulphur bridge between two alanine residues, is a constituent of some animal proteins[1]; it is also found in hydrolysates of wool and hair treated with alkali[2,3]. The literature contains only a few references to polymerization of tetrafuntional amino acids.

Since cystine is more easily accessible than lantionine, it seemed worthwhile to start preliminary experiments in this direction with cystine derivatives, and to try to obtain polylanthionine derivatives from the polycystine preparations formed. However, this conversion was not satisfactory and the final polymers were not pure.

To get structurally defined polymeric polypeptides of lanthionine the following synthetic route was chosen: carbobenzoxy amino acrylic acid was esterified with benzyl alcohol and L-cysteine was then added to the double bond of the acrylic ester. In this way lantionine was formed in which one carboxyl and the adjacent amino group were protected and only two free functional groups remained for polymerization. The polymer was obtained via the N-carboxy anhydride, prepared by the Fuchs-Farthing method[4,5]. The polymerization was carried out in pyridine solution.



According to the above scheme, the mono-benzyl ester mono-carbobenzoxy lanthionine obtained has two asymmetric carbon atoms, the protected side being racemic and the unprotected having the L-configuration. This leads to the formation of two diastereoisomers, the separation of which was achieved by means of their different solubilities.

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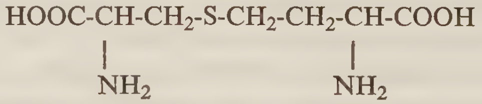
4. Fuchs, F., 1922, *Ber.* **55**, 2943.
5. Farthing, A. C., 1950, *J. Chem. Soc.*, 3213.

Syntheses of polymeric polypeptides of cystathionine

Max Frankel and David Gertner, *Department of Organic Chemistry, The Hebrew University of Jerusalem*



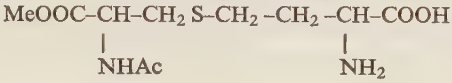
Cystathionine (I) $\underset{NH_2}{HOOC\text{-}CH}\text{-}CH_2\text{-}S\text{-}CH_2\text{-}CH_2\text{-}\underset{NH_2}{CH}\text{-}COOH$ is a tetrafunctional di-amino di-carboxylic acid, containing a sulphur atom between an alanine residue and an α-amino butric acid residue. It is supposed to be an intermediate in the formation of cysteine from serine by transthiolation. Due to its asymmetry, the preparation of defined polymeric polypeptides of cystathionine is more complicated than in the case of lanthionine[1].

An approach to synthesis of defined polymeric polypeptides of cystathionine was made by using as a monomer a cystathionine derivative in which the two adjacent reactive functions at one side of the molecule were protected, while at its other end the amino and carboxyl groups remained free for polymerization. The protective groups can be introduced either at the alanine side, giving on polymerization a polycystathionine representing a poly-homocysteine derivative; or on the side of the α-amino butyric acid, yielding after polymerization a polycystathionine, which is a polycysteine derivative.

The two different protected monomers were prepared as follows:

(a) Mono methyl ester mono acetyl cystathionine (II)

(II)

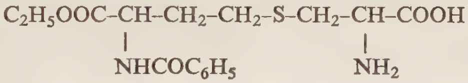


$$MeOOC-\underset{NHAc}{\underset{|}{CH}}-CH_2\,S-CH_2-CH_2-\underset{NH_2}{\underset{|}{CH}}-COOH$$

was synthesised by adding acetamino methyl acrylate to homocysteine. The functional groups, remaining free for the intended polymerization, belong to the α-amino butyric acid unit.

(b) Mono-ethyl ester mono-benzoyl cystathionine (III)

(III)

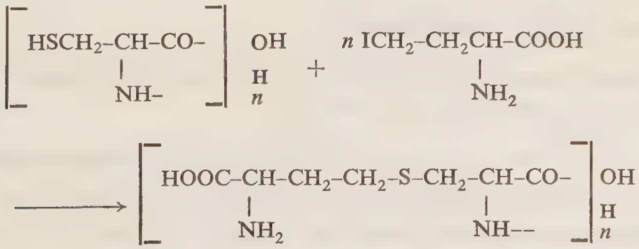


$$C_2H_5OOC-\underset{NHCOC_6H_5}{\underset{|}{CH}}-CH_2-CH_2-S-CH_2-\underset{NH_2}{\underset{|}{CH}}-COOH$$

was synthesised by condensing α-benzoyl amino-γ-iodo ethyl butyrate with cysteine. Here the free functional groups are situated at the alanine side of the molecule.

In both cases, polymers were prepared via the N-carboxy anhydrides, which were obtained by the Fuchs-Farthing method, and polymerized in pyridine solution for 15 hrs. at room temperature and 2 hrs. in a boiling water bath.

(c) In addition, a completely different route was used in the synthesis of polycystathionine, consisting in the reaction of γ-iodo α-amino butyric acid with polycysteine which is already known[2].



$$\left[HSCH_2-\underset{NH-}{\underset{|}{CH}}-CO-\right]\begin{matrix}OH\\H\\n\end{matrix} + n\,ICH_2-CH_2\underset{NH_2}{\underset{|}{CH}}-COOH \longrightarrow \left[HOOC-\underset{NH_2}{\underset{|}{CH}}-CH_2-CH_2-S-CH_2-\underset{NH--}{\underset{|}{CH}}-CO-\right]\begin{matrix}OH\\H\\n\end{matrix}$$

The reaction product obtained is a polycystathionine which is a polycysteine derivative and has free amino and carboxyl groups at the α-amino butyric acid side.

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2. Berger, A., Naguchi, J. and Katchalski, E., 1956, *J. Am. Chem. Soc.*, 78, 4483.

The telomerization of ethylene with ethyl bromoacetate. Part II. The preparation of linear ω-bromocarboxylic acid esters

A. Baniel, L. M. Shorr, M. Rogozinski* and A. Varsanyi, *Israel Mining Industries, Haifa*

The telomerization of ethylene under pressure with ethyl bromoacetate in the presence of free radical initiators yields the esters of ω-bromocarboxylic acids[1]. The factors influencing the reaction, as well as a description of the micro pressure reactor used for its examination, will be presented.

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1. Baniel, A., Shorr, Leonard and Israel Mining Industries, *Israeli Patent* 10,830 (Jan. 22, 1959); 1959, *C.A.* 53, 12185e.

The telomerization of ethylene with ethyl bromoacetate. Part I.
The phase equilibrium of the reactants at elevated pressures

L. M. Shorr, M. Rogozinski,* A. Varsanyi and A. Baniel, *Israel Mining Industries, Haifa*

The telomerization products obtained from the reaction of a telogen with an olefin are in large part a function of the relative concentrations of the reagents. An examination of the system ethyl bromoacetate — ethylene revealed that the solubility relationships vary sharply with slight changes of pressure. The complex phase equilibrium which was subsequently evolved indicated the conditions under which reactant ratios could be varied most easily.

Third Session Chairman: D. Samuel

On the Oumnova acyloin rearrangement

Shalom Sarel* and Avraham Friedlender, *Department of Pharmaceutical Chemistry, The Hebrew University School of Pharmacy*

We were unable to effect the conversion of α-hydroxy-ketones into isomeric hydroxyketone by means of alcoholic sulphuric acid, as claimed by Oumnova[1]. We found, however, that such a rearrangement could be materialized by a variety of basic reagents such as alkali hydroxides and aluminium alkoxides. By use of aluminium isopropoxide as a catalyst, the acyloin rearrangement is accompanied by a concurrent reduction reaction, leading ultimately to the formation of a mixture of isomeric glycols, as described below.

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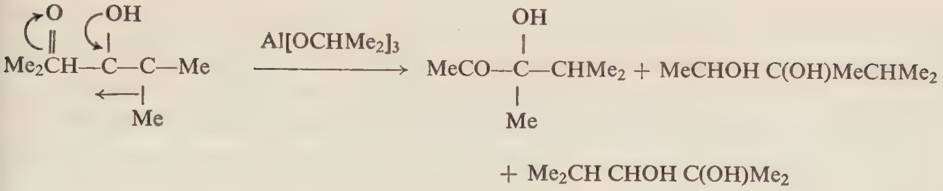

$$Me_2CH{-}\overset{\overset{O}{\|}}{C}{-}\underset{\underset{Me}{|}}{\overset{\overset{OH}{|}}{C}}{-}Me \xrightarrow{Al[OCHMe_2]_3} MeCO{-}\underset{\underset{Me}{|}}{\overset{\overset{OH}{|}}{C}}{-}CHMe_2 + MeCHOH\,C(OH)MeCHMe_2 + Me_2CH\,CHOH\,C(OH)Me_2$$

The mechanism and the steric requirements of this reaction will be discussed.

REFERENCE
1. Oumnova, A. I., 1928, *Bull. soc. chim.*, (*France*), 43, 568.

The Grignard reaction of cyclic carbonates

Shalom Sarel, Leo A. Pohoryles* and Dina Marcus, *Department of Pharmaceutical Chemistry, The Hebrew University School of Pharmacy*

Following a recent study aimed at establishing the location of bond-fission and structural effects upon rates of hydrolysis of cyclic carbonates[1,2], the Grignard reaction of these esters was undertaken. The present communication is concerned with a study of structural effects both in the Grignard reagent and the cyclic carbonates upon the course and the nature of reaction products. Depending upon chemical structure of both reagents and reaction conditions the reverse addition of Grignard reagents leads to the formation of a mixture consisting of the expected hydroxy-ester, halo hydrine and the corresponding carboxylic acid, in different ratios. In analogy with carboxylic esters we found that the addition of the Grignard reagent to substituted cyclic carbonates is affected by the structure of the ester in a way which parallels that observed in their alkaline hydrolysis. The results obtained in this study will be discussed in terms of steric effects.

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The deiodination reaction of *o*-iodo anisole

M. Anbar and R. Rein*, *Department of Isotope Research, The Weizmann Institute of Science*

The rate of deiodination of *o*-iodo anisole has been investigated in acetic acid as solvent in presence of increasing concentrations of H_2SO_4 and in presence of Cl . The rate was found proportional to h_o,which implies a rapid pre-equilibrium with a proton.

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* Lecturer

Next it was found to be catalysed by chloride ions, which means that the iodine abstraction is base catalysed. On the other hand the rate of iodine exchange with *o*-iodo anisole[1] was found to be unaffected by H_2SO_4 and it is faster than the rate of deiodination at low acidities. The implications of these results compared with the mechanisms of iodination[2a,b] and of hydrogen exchange with anisole[3] will be discussed.

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1. Anbar, M. and Rein, R., 1959, *Bull. Res. Counc. of Israel*, **8A**.
2a. Painter, B. S. and Soper, F. G., 1947, *J. Chem. Soc.*, 342.
2b. Growenstein, E. and D. Kilby, *J. Am. Chem. Soc*, **79**, 2972.
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The reaction of acetylene with solutions of bromine in tetrabromoethane (TBE)

L. M. Shorr*, H. Cohn and A. Varsanyi, *Israel Mining Industries, Haifa*

Acetylene reacts with bromine in tetrabromoethane solutions containing 100 g/1 of the halogen to form both acetylene dibromide and tetrabromoethane. An investigation of the factors influencing the reaction as well as the mechanism by which both of these compounds are produced was the basis for the synthesis of tetrabromoethane in the I.M.I. process.

The nitric acid oxidation of *iso*-amyl alcohol

L. M. Shorr and J. Segall*, *Israel Mining Industries, Haifa*

The nature of the decomposition which occurs in 1 to 3 N nitric acid solutions in iso-amyl alcohol[1] was examined. The products obtained are those resulting from the oxidation of the alcohol followed by esterification. Thus, esters such as iso-amyl formate and iso-amyl nitrite have been recovered. A description and discussion of these reactions will be given.

REFERENCE

1. Used in the I.M.I. process for the conversion of KCl to KNO_3
Cf: Baniel, A., Blumberg, R. and Israel Mining Industries, *Israeli Patent* 9660 (June 6, 1957)

The photochemical decomposition of tetrabromoethane (TBE) in the presence and absence of iron

L. M. Shorr* and J. Segall, *Israel Mining industries, Haifa*

Pure tetrabromoethane decomposes upon exposure to light and oxygen, yielding pentabromoethane and tribromoethylene predominantly. However, in the presence of iron these decomposition products are almost completely absent and acetylene dibromide is formed almost exclusively. A mechanism which explains the formation of the several products will be presented.

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* Lecturer

Fluoro-analogs of mevalonic acid and related compounds

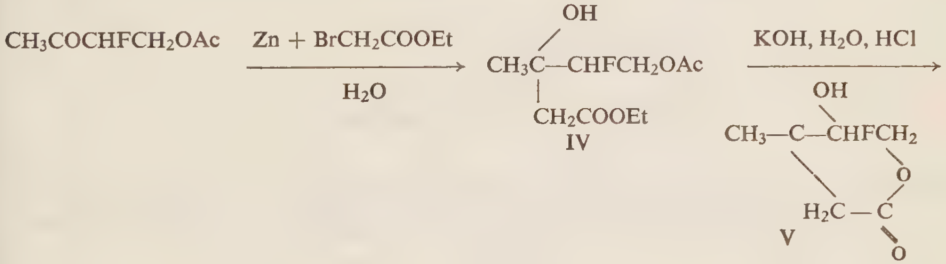
SASSON COHEN* AND E. D. BERGMANN, *Israel Institute of Biological Research, Ness Ziona*

In this note we report the synthesis of some fluoro-analogs of mevalonic acid and related compounds involved in the biosynthesis of cholesterol[1]. The reaction of ethyl fluoroacetate with ketones, in presence of sodium hydride, gives simultaneously 1-fluoro-2, 4-diketones (I) and α-fluoro-β-hydroxy esters (II):

$$FCH_2COOEt + CH_3COR \xrightarrow{NaH} \underset{(I)}{FCH_2COCH_2COR} + \underset{(II)}{CH_3C(R)(OH)CHFCOOEt}$$

The corresponding acids are readily obtained from II by hydrolysis; when R = $-CH_2CH_2OAc$, the δ-lactone is formed.

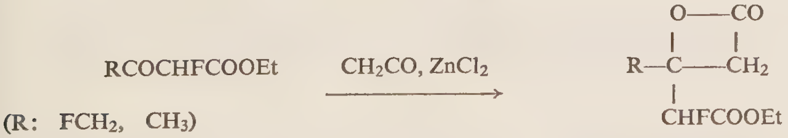
Fluoroacetone and formaldehyde react at *p*H 8–8.5 to yield $CH_3COCHFCH_2OH$ (III) rather than $FCH_2COCH_2CH_2OH$; after acetylation, III affords upon reaction with zinc and ethyl bromoacetate, the hydroxy ester IV, which can be smoothly saponified to the δ-lactone *V*:



IV

V

The $ZnCl_2$-catalysed addition of ketene to $RCOCH_2CEOOt$ (R: CH_3, FCH_2, CF_3) gave O-acylated acetoacetates only; however, RCOCHFCOOEt gave β-lactones:



These β-lactones were hydrolysed with base, but the expected β-hydroxy-β-methylglutaric acids failed to crystallize. These and related reactions will be discussed.

REFERENCE

1. For a review on the subject, see Cornforth et al., 1959, *Tetrahedron*, 5, 311.

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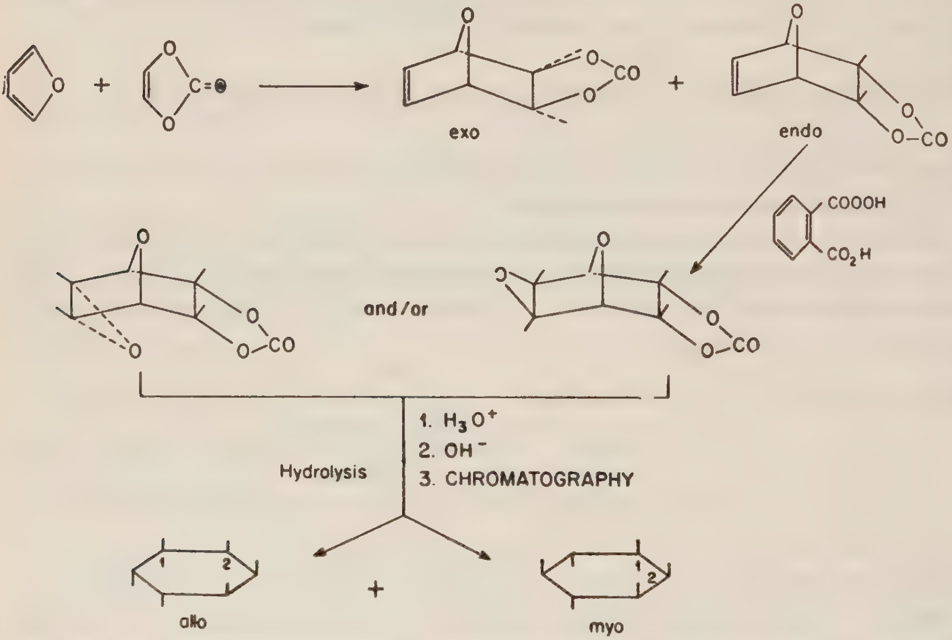
* Lecturer

Fourth Session Chairman: Z. Falchowicz

A stereospecific total synthesis of inositols

Shalom Sarel* and Hanna Kowarsky, *Department of Pharmaceutical Chemistry, The Hebrew University School of Pharmacy, Jerusalem*

Although a few members of the inositols have already been partially synthetized,[1] actually no total synthesis of any member in this series has been recorded. This communication reports a new three-step synthesis of either *myo-* or *allo-*inositols involving stereospecific reactions, as depicted below:



An account of product studies, as well as the stereochemistry and mechanism of reactions reported here, will be described.

REFERENCE

1. Angyal, S. J., 1957, *Quart. Reviews*, **11**, 212.

The structure of the cucurbitacins

Youval Shvo* and David Lavie, *The Weizmann Institute of Science, Rehovot*

The cucurbitacins are a group of compounds isolated from different species of the cucurbitaceae; they have been found to be tetracyclic triterpenes and structures have been proposed[1,2] for four of them, namely, elatericin A, $C_{30}H_{44}O_7$, elatericin B

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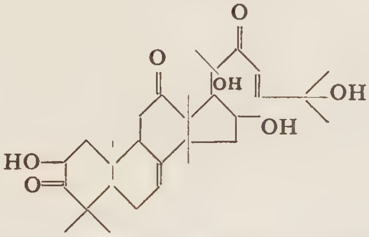
* Lecturer

$C_{30}H_{42}O_7$, cucurbitacin B, $C_{32}H_{46}O_8$ and elaterin, $C_{32}H_{44}O_8$[3]. However, in these structures the location of one carbonyl group and a double bond, known to be inert, were left undetermined. By a sequence of successive degradations made on elatericin A, we have now obtained products whose nature allow us to locate the remaining two functions in the molecule, thereby presenting a full structure. These two functions were located at C 12 for the carbonyl group and at the position 7–8 for the double bond in the trimethylated steroid skeleton. We have previously[1] assumed, on spectroscopic grounds, that the carbonyl group was at C 11. The degradation products which will be presented and described have been obtained by oxidation of elatericin A with periodic acid, thereby severing the side chain and cleaving ring A. Bromination of the allylic position of the double-bond with N.B.S. and subsequent dehydrobromination formed a conjugated diene. This new product will be presented and its characteristics discussed for the location of the double bond. Osmium tetroxide hydroxylation of the degraded molecule led to a new glycol system which was oxidized to a mono-keto-alcohol; dehydration of this compound yielded a 1,5 conjugated diketone in the enolized form[4], the structure of which will be discussed. This sequence of reactions led to the proposed full structure (I) for elatericin A.



Owing to the interrelationship already described[3] among the four compounds investigated, their full structures can now be established.

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1. Lavie, D. and Shvo, Y., 1959, *Chemistry and Industry*, 429.
   Lavie, D. and Willner, D., 1959, *Proc. Chem. Soc.*, 191.
2. Enslin, P. R., Rehm, S. and Rivett, D. E. A., 1957, *J. Sci. Food Agric.*, **8**, 673.
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4. Lenhard, R. H. and Bernstein, S., 1956, *J. Am. Chem. Soc.*, **78**, 989.

6-Thiouric acid and some of its derivatives

Gershon Levin, Felix Bergmann* and Abraham Kalmus, *Department of Pharmacology, The Hebrew University–Hadassah Medical School, Jerusalem*

6-Thiouric acid (IIIa) has become of great interest as a metabolite of 6-mercaptopurine which has been introduced for the treatment of leucemia. IIIa has been synthesized recently by direct thiation of uric acid, a process which involves preferentially carbon

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* Lecturer

6 as in xanthine[1,2]. However, the product so obtained is contaminated with other sulphur derivatives of uric acid and purification requires the use of anion exchangers, resulting in very modest yields.

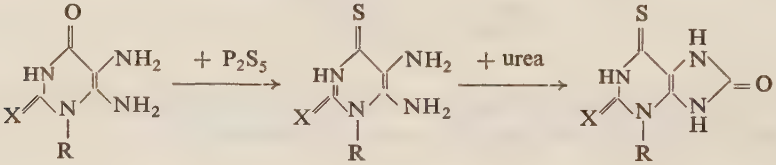
We have now observed that in 5,6-diaminopyrimidines of type I, the difference in reactivity of position 2 and 4 toward P_2S_5 is even more pronounced, and have thus obtained compounds II, which can be cyclized to the corresponding uric acids (III) in very high yields and in excellent state of purity. The properties of the 6-thiouric acids are summarized in Table I.

TABLE I

Physical properties of 6-thiouric acid and its derivatives

Compound	$\lambda_{max.}$ (mμ) at *p*H 8.0	R_F^a	Fluorescence
6-Thiouric acid (IIIa)	245 346	0.28	Blue
3-Methyl-6-thiouric acid (IIIb)	247 357	0.40	Blue
2,6-Dithiouric acid (IIIc)	245 283 357	0.44	Blue
3-Methyl-2,6-dithiouric acid (IIId)	255 281 364	0.57	Violet

(a) Solvent used: Isopropanol, 65 vols.; dimethyl-formamide, 25 vols.; water, 10 vols.



I II III

a: X=O; R=H b: X=O; R=CH_3 c: X=S; R=H
d: X=S; R=CH_3

REFERENCES

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Synthesis and properties of 3-methylpurines

Felix Bergmann, Gershon Levin*, Abraham Kalmus and Hanna Kwietny
Department of Pharmacology, The Hebrew University–Hadassah Medical School, Jerusalem

In 3-methylpurines, the distribution of double bonds in the pyrimidine ring is fixed. This makes it possible to draw conclusions about the structure of other purines either in solution or in an activated enzyme – substrate complex. The following derivatives have been synthesized:

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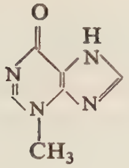

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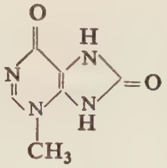
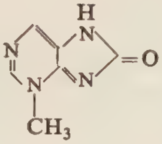
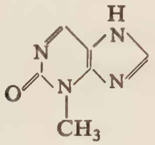


II



III



IV

The synthesis of 3-methylpurine, however, which was attempted by a variety of methods, failed. The properties of the 3-methyl derivatives are compared in Table I with those of the unsubstituted parent substances.

TABLE I

Comparison of purines with their 3-methyl derivatives.

Purine	λ_{max} (mμ) of neutral molecule	R_F in solvent A**	R_F in solvent B***
1. Hypoxanthine	251	0.47	0.40
3-Methylhypoxanthine (I)	264	0.35	0.33
2. 6,8-Dihydroxypurine	256	0.30	0.34
3,6-Dihydro-3-methyl-6-oxo-8-hydroxypurine (II)	274	0.20	0.06
3. 8-Hydroxypurine	275	0.55	0.63
3-Methyl-8-hydroxypurine (III)	299	0.42	0.41
4. 2-Hydroxypurine	314	0.25	0.22
2,3-Dihydro-2-oxo-3-methylpurine (IV)	318	0.48	0.39

** Solvent A: 95% ethanol — 85ml; acetic acid — 5ml; H_2O — 10ml.
*** Solvent B: 95% ethanol — 70ml; pyridine — 20ml; H_2O — 10ml.

The following general conclusions can be drawn:

1. Fixation of a double bond in position 1–2 produces a bathochromic shift, much greater than can be ascribed to methylation as such (compare I–III with IV).
2. Fixation of a double bond in position 1–2 endows the compounds with R_F values *smaller* than for the non-methylated purines, in contrast to all other known methylpurines (compare again I-III with IV).

Conformational analysis by means of dipole moments and spectra

Ernst D. Bergmann* and S. Yaroslavsky, *Department of Organic Chemistry, The Hebrew University of Jerusalem*

The dipole moments, ultraviolet and infrared spectra of the following cyclohexanone derivatives have been measured: 4-phenyl (moment 2.68 D), 2-bromo-4-phenyl (2.83), 2-bromo-4-cyano-4-phenyl (4.07), 4-cyano-4-phenyl (3.83), 4-(*p*-dichlorophenyl)-4-cyano (2.73); 2-bromo-2-(*p*-chlorophenyl) (4.36) (all dipole measurements in benzene at 30°C).

The measurements permit an exact determination of the conformation of the compounds and, therefore, the elimination of certain doubtful points which have recently arisen in the evaluation of the spectra of such compounds. Some of the results were unexpected and, therefore, of specific interest, e.g.: in 2-bromo-4-cyano-4-phenylcyclohexanone the bromine is equatorial, *cis* to the phenyl and *trans* to the nitrile group; in 2-bromo-4-phenylcyclohexanone we have a mixture of a chair form with axial bromine, *trans* to the (equatorial) phenyl, and of a boat form with equatorial bromine, *trans* to the equally equatorial phenyl. 2-Bromo-2-phenylcyclohexanone consists of a mixture of about 60% of the form with equatorial bromine and axial phenyl and 40% of the form with axial bromine and equatorial phenyl.

From the temperature dependency of the dipole moment of 2-chlorocyclohexanone, one must also conclude that equatorial bromine is more stable than axial bromine.

Different cross-conjugated chromophores

Samuel Yaroslavsky*, *Department of Chemistry, Technion–Israel Institute of Technology, Haifa*

Branch *et al.*[1] have shown that 4′-dimethylamino-chalcone exhibits two distinct ultra-violet bands which may be ascribed to the absorption of the two individual chromophores as exemplified in compounds like 4-dimethylaminoacetophenone and benzalacetone. A similar case was that of 4-dimethylaminochalcone. Other cases of different cross-conjugated chromophores (D.C.C.C.), simplified by elimination of any auxochromic group, have been examined by us. Thus 5-benzylidene-3-phenyl-2-cyclopentenone shows two separate bands at 240mμ (ε 15,500) and 325mμ (ε 23,800). Similarly 5-benzyldene-2-cyclopentylidene-cyclopentanone absorbs at 237mμ (ε 10,500) and 330mμ (ε 33,000). It should be noted that while the absorption of the chromophore of the longer wave-length is shifted bathochromically when cross-conjugated, that of the chromophore of the shorter wave-length is shifted hypsochromically, as summarized in Table I.

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* Lecturer

TABLE I

Contradicting ultra-violet shifts of different cross-conjugated chromophores

Compound	First band	Absorption of individual chrom.	$\Delta\lambda$	Second band	Absorption of individual chrom.	$\Delta\lambda$
5-Benzylidene-3-phenyl-2-cyclopentenone	240	281[2]	—41	325	298[3]	+27
5-Benzylidene-2-cyclopentylidene--cyclopentanone	237	253[4]	—16	330	298[3]	+32
5-Benzylidene-4-hydroxy-3,4-diphenyl-2-cyclopentenone[5]	268	281[2]	—13	330	298[3]	+32

Such contradictory shifts are found in many other cases of D.C.C.C., including triphenylmethane dyes, which in their quinonoid form may be looked upon as D.C.C.C. Other dyes and organic compounds will be discussed and a generalization of the phenomenon will be attempted. Also a comparison will be made between D.C.C.C. and equal cross-conjugated chromophores including their respective derivatives.

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Fifth Session Chairman: F. SONDHEIMER

Conjugative transmissions in the vinycyclopropane systems. II. Cyclic addition of malcic anhydride to α-cyclopropylstyrene

SHALOM SAREL* AND ELI BREUER, *Department of Pharmaceutical Chemistry, The Hebrew University School of Pharmacy*

The present communication presents new evidence showing that vinylcyclopropane can enter into 1,5-conjugative cyclic addition reaction when it is properly activated, for example by introducing a phenyl substituent. It will be shown that α-cyclopropylstyrene (I) is able to add maleic anhydride by two different routes under the same conditions. The first, *a*, proceeds in the 1,4-manner, giving a bis-adduct II, whereas the second, *b*, presumably occurs in the 1,5-manner, providing a new seven-membered ring compound, III. While the first requires conjugation of the exo-cyclic double-bond in I with the aromatic nucleus, the second probably involves electronic interactions between cyclopropane and the exo-cyclic double-bond. An account of the physical and chemical evidence for the structual assignments for II and III will be given.

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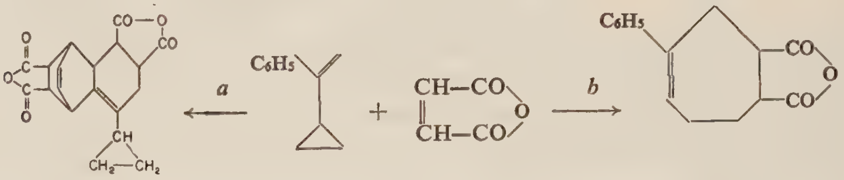
* Lecturer



II I III

Iodo- and bromo-lactones

Joseph Klein, *Department of Organic Chemistry, The Hebrew University of Jerusalem*

The reaction of iodine with a salt of cyclohexene-3-carboxylic acid (I) gives a γ-lactone in which the iodine is located in the 4-position and *trans* to the lactone ring. Structure and configuration were proven by determination of the dipole moment and the reaction with potassium hydrogen carbonate, which leads to the corresponding epoxide. The reaction of bromine with a salt of (I) yields the analogous bromolactone.

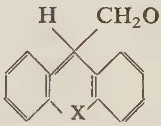
The free acid (I) gives with bromine a dibromide which is converted by sodium hydrogen carbonate into an isomeric bromolactone (bromine *cis* to the lactone ring). Dipole moment measurements establish the configuration of the two isomers.

Similar lactones have been obtained from 2-cyclohexene-1-carboxylic acid and 2- and 3-cyclohexene-1-acetic acids.

Some ring enlargement reactions

Ernst D. Bergmann and Mordecai Rabinowitz*, *Department of Organic Chemistry The Hebrew University of Jerusalem*

Compounds of the general formula (I) undergo dehydration (and their p-tosylates, solvolysis), which is accompanied by ring enlargement, so that products of the general formula (II)



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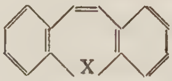

II

(X=O,NR, S, CHR)

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* Lecturer

are formed. For the case X = O, this reaction has been known[1]; it has now been studied for the transformation of (1) 9-hydroxymethyl-thioxanthene (I, X = S) (m.p. from cyclohexane, 110–111°), into dibenzo [bf] thiepin (II, X = S) (m.p. from methanol, 90°);

(2) 9-hydroxymethyl-9,10-dihydroacridine (I, X = NH) (m.p. from petroleum ether, 135–136°) into dibenzo [bf] azepin (II, X = NH) (m.p. 190–192°);

(3) *cis*-9,10-dihydroxymethyl-9,10-dihydroanthracene (I, X = $CH.CH_2OH$) (m.p. from benzene, 166°) into 1-hydroxymethyl-2,3,6,7-dibenzocyclohepta-2,4,6-triene (II, X = $CH\text{-}CH_2OH$) (m.p. from ligroin, 88°).

In the last case, the rearrangement to some extent takes place twice, yielding 3,4,7,8-dibenzocyclo-octa-1,3,5,7-tetraene.

The properties of the substances will be discussed.

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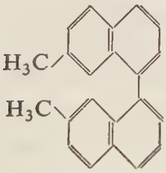
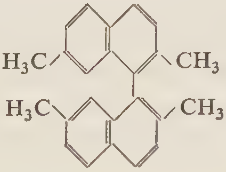
Syntheses with phenyl lithium

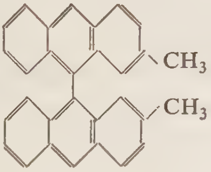
Ernst D. Bergmann and Perez Bracha*, *Department of Organic Chemistry, The Hebrew University of Jerusalem*

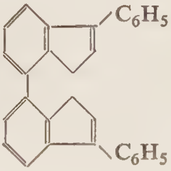
In the manner known for the conversion of benzyl bromide to dibenzyl by phenyl lithium, cinnamyl bromide yields 1,6-diphenylhexa-1,5-diene, which can be dehydrogenated by chloranil to 1,6-diphenylhexa-1,3,5-triene. 3,3-Biphenylene-allyl bromide (m.p. 93°) gives 1,1,6,6-dibiphenylenehexa-1,5-diene and, by dehydrogenation, dibiphenylene-hexatriene. 1-Bromo-5-phenylpenta-2,4-diene was prepared starting with ethyl cinnamenyl-acrylate, but proved too unstable to undergo the above "dimerisation" reaction.

The extension of this method, which has been applied so successfully to the synthesis of complex phenanthrene derivatives, to compounds of the following type, will be reported:









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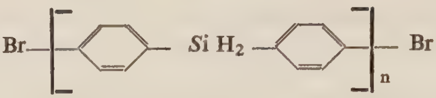
* Lecturer

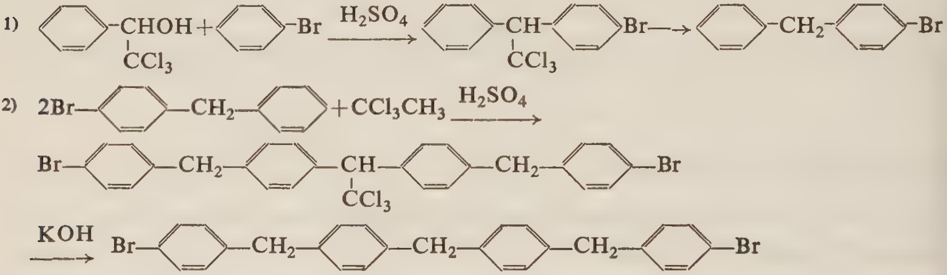
Higher homologues of diphenylmethane

Arjeh Galun, *Atomic Energy Laboratories*

In order to study the influence of silicium in silane-type polymers, such as



we synthesized the carbon-analogs of these polymers. The simplest method for the preparation of the desired compounds is the condensation of chloral or trichloromethyl-phenyl-carbinol with an aromatic halide or hydrocarbon in the presence of sulphuric acid, in analogy to the preparation of D.D.T. One obtains derivatives of trichloro-diphenylethane, from which the desired compounds are derived by hydrolysis and decarboxylation. The following reaction schemes are representative:



Also the first dihalide of this series, di-(*p*-bromophenyl)-methane, which has been obtained before by direct bromination of diphenylmethane, can better be prepared by condensation of trichloromethyl-(*p*-bromophenyl)-carbinol and bromobenzene.

The reaction conditions are dictated by the starting materials; some react already at room temperature, whereas others require prolonged heating, or show a tendency towards sulphonation. Hydrolysis and decarboxylation take place simultaneously when the products are heated with alkali in diethylene glycol.

Model reactions and attempted synthesis of a potential pentalene system

A. Modiano and Michael Cais*, *Technion-Israel Institute of Technology, Haifa*

Numerous attempts have been reported[1] for the synthesis of the still unknown hydrocarbon pentalene, I.

Our experience with the chemistry of cyclopentadienyl manganese tricarbonyl,

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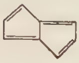
* Lecturer

II[2–4] has suggested to us that II might provide a suitable starting material for a novel approach towards the synthesis of a potential pentalene system.

The synthesis of the model compounds, III and IV will be reported. The attempted synthesis of V will be discussed.



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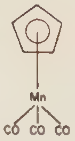

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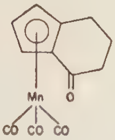
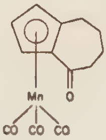
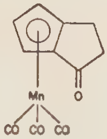


III



IV



V

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A novel synthesis of 6,7-benzocoumaran-2-ones

Samuel Yaroslavsky* and Ernst D. Bergmann, *Department of Organic Chemistry, The Hebrew University of Jerusalem*

A new type of ring-closure, characterized by the formation of a naphthalene nucleus connected to a γ-lactone ring (6,7-benzocoumaran-2-ones), is described. It seems to be general for acid-esters, acid-lactones and dicarboxylic acids derived from the Stobbe products of benzyl ketones. For example, the product (I) of the Stobbe condensation between 2-phenylcyclohexanone and diethyl succinate was converted by means of polyphosphoric acid into 4,5-cyclohexeno-6,7-benzocoumaran-2-one (II). The dicarboxylic acid (III) obtained from the Stobbe product of phenylacetone by basic hydrolysis was cyclized to 4-methyl-6,7-benzocoumaran-2-one (IV). The lactonic acid (V) derived from the Stobbe product of 2-phenylcycloheptanone by acid hydrolysis was cyclized to 4,5-cyclohepteno-6,7-benzocoumaran-2-one (VI). More complex examples will be reported and a possible mechanism will be proposed.

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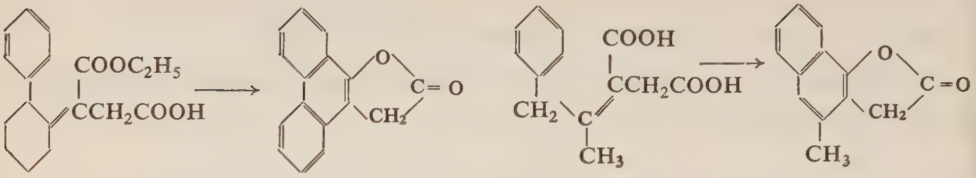
* Lecturer



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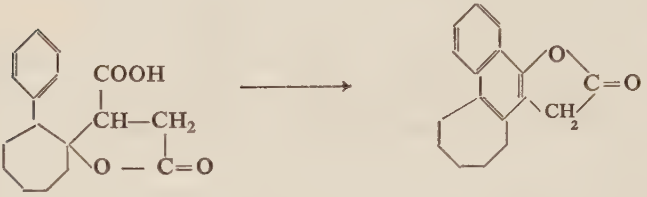
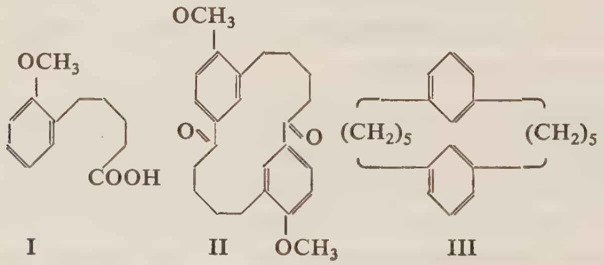


V VI

Intermolecular cyclization by polyphosphoric acid. The preparation of (5:5) metacyclophane

Shlomo Bien, *Technion – Israel Institute of Technology, Haifa*

It was shown that polyphosphoric acid cyclization of δ-(2-methoxyphenyl)-valeric acid (I) gave a mixture of high melting neutral compounds. By chromatographic separation the dimer (II) was obtained and its structure proved by $KMnO_4$ oxidation. The compound could be transformed to the unknown "metacyclophane" (III).



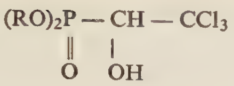
I II III

Sixth Session Chairman: M. Sela

Reaction of dialkyl thiophosphites with chloral

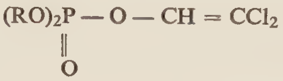
Zvi Pelchowicz, *Israel Institute for Biological Research, Ness Ziona*

The reaction between dialkyl phosphites and chloral is known to lead to very important insecticides of the general formula



$$(RO)_2\underset{\underset{O}{\|}}{P}-\underset{\underset{OH}{|}}{CH}-CCl_3$$

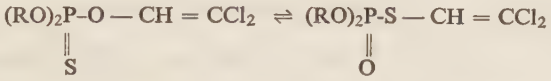
These compounds undergo, upon treatment with alkali, not only dehydrohalogenation, but also a rearrangement, and substances of the formula



$$(RO)_2\underset{\underset{O}{\|}}{P}-O-CH=CCl_2$$

are biologically even more active.

In the reaction of chloral with dialkyl thiophosphites, analogs of the last-mentioned phosphate esters are formed directly:



$$(RO)_2\underset{\underset{S}{\|}}{P}\text{-}O-CH=CCl_2 \rightleftharpoons (RO)_2\underset{\underset{O}{\|}}{P}\text{-}S-CH=CCl_2$$

This formula was proven by the infrared spectra and the molar refraction of the compounds.

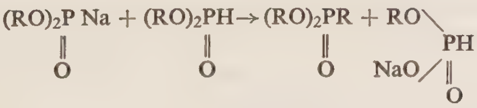
The reaction mechanisms involved as well as some toxicological data will be discussed in the lecture.

Alkylation of the sodium salts of dialkyl phosphites by dialkyl phosphites

Zvi Pelchowicz and Sarah Brockson-Fuchs, *Israel Institute of Biological Research, Ness Ziona*

One of the methods for the preparation of dialkyl alkylphosphonates is the Arbuzov-Michaelis reaction, the reaction of alkyl halogenides with the sodium salts of dialkyl phosphites. This reaction gives satisfactory yields, but a careful study revealed that some undesirable side reactions also occur.

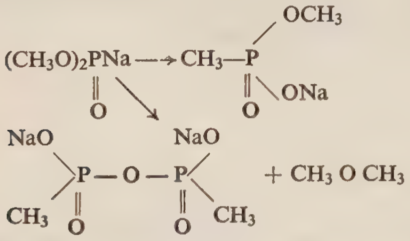
In an investigation of these side reactions it was found that the preparation of the sodium salts of the dialkyl phosphites is accompanied by a homogeneous reaction, in which dialkyl alkylphosphonates are formed according to the following equation:



$$(RO)_2\underset{\underset{O}{\|}}{P}\,Na + (RO)_2\underset{\underset{O}{\|}}{P}H \rightarrow (RO)_2\underset{\underset{O}{\|}}{P}R + \begin{matrix} RO\diagdown \\ \quad PH \\ NaO\diagup\| \\ \quad O \end{matrix}$$

Development of this reaction led to a method for the preparation of esters of alkylphosphonic acids and for the synthesis of the sodium salts of monoalkyl phosphites.

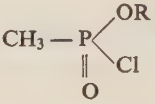
The reaction was studied for the sodium salts of diethyl, dipropyl and di-*n*-butylphosphite; in the case of the sodium salt of dimethyl phosphite, an unexpected reaction took place. This sodium salt undergoes immediately an exothermic reaction which is accompanied by the liberation of dimethyl ether and gives a salt mixture according to the following scheme:



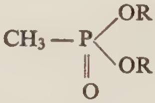
Methods for the synthesis of *o*–alkyl-methylphosphonyl chlorides

Zvi Pelchowicz, *Israel Institute for Biological Research, Ness Ziona*

The compounds of the general formula



are of importance as starting materials for the synthesis of insecticides. We have found that the acid hydrolysis of the diesters



leads stepwise to the corresponding monoesters and free acids. This observation led us to expect that the diesters could also be monochlorinated and would thus give the desired products. Indeed, thionyl chloride and, better, oxalyl chloride gives only the ester chlorides of methylphosphonic acid.

The analogous reaction of dialkyl methylphosphothionates also leads to the ester chlorides of methylphosphonic acid, accompanied by elementary sulphur. The same effect was observed in the reaction of the sulphur compounds with phosphorus pentachloride, at least as the main path of reaction.

The mechanisms of these reactions will be discussed in the lecture.

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* Lecturer

Properties of diethyl oxalofluoroacetate

Ernst D. Bergmann* and Israel Shahak, *Department of Organic Chemistry, The Hebrew University of Jerusalem*

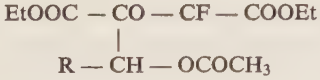
The title compound is of considerable importance as starting point for the synthesis of compounds of biological interest. In its preparation from diethyl oxalate and ethyl fluoroacetate[1], no yields higher than 55 % could be achieved, as the condensation proved reversible and fairly large amounts of the crystalline sodio-enolate of fluoroacetate remain in the (solid) condensation product.

1) Diethyl sodio-oxalofluoroacetate (I) reacts with acid chlorides (or anhydrides) to give C-acyl derivatives ROOC—CO—CF(Ac)—COOEt, the reactions of which will be reported. Their structure follows, *inter alia*, from the acidic hydrolysis to C-acyl-fluoropyruvic acids HOOC—CO—CHF—Ac.

2) (I) (surprisingly, not the free ester) reacts with bromine to give ethyl bromoethoxalyl-fluoracetate ROOC—CO—CFBr—COOR, which contains "positive" bromine and is hydrolysed to bromofluoropyruvic acid.

3) Condensation of diethyl oxalofluoroacetate with ethyl cyanoacetate gives the normal condensation product which, upon acidic hydrolysis, loses the cyano-group and yields the interesting triethyl fluoroaconitate.

4) In the same manner as the non-fluorinated parent compound, diethyl oxalofluoroacetate reacts with aliphatic aldehydes RCHO in the presence of acetic anhydride and sodium acetate, to give products of the general formula



$$\begin{array}{c}\text{EtOOC}-\text{CO}-\text{CF}-\text{COOEt}\\ |\\ \text{R}-\text{CH}-\text{OCOCH}_3\end{array}$$

which, upon acidic hydrolysis, give the corresponding substituted pyruvic acids.

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Fluorine-containing polycyclic compounds of the carcinogenic series

Ernst D. Bergmann and Jochanan Blum*, *Department of Organic Chemistry, The Hebrew University of Jerusalem*

The purpose of the present study was to explore whether fluorine-substitution of the K-region of carcinogenic polycyclic aromatics would enhance or destroy the biological activity, and thus to examine the validity of current theories of carcinogenic action.

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* *Lecturer*

1) By condensation of 4-fluoro-1-naphtylamine and *p*- and *m*-toluidine and subsequent cyclisation, 2,9- and 3,9-dimethyl-7-fluoro-5,6-benzacridine (m.p. 174° and 172°, respectively) were prepared.

2) Starting from the reaction between phthalic anhydride and 4-fluoronaphthylmagnesium bromide, 3-fluoro-9-methyl-1,2-benzanthracene, m.p. 54°, and 3-fluoro-9,10-dimethyl-1,2-benzanthracene, m.p. 94°, were obtained. Analogously, through the Friedel-Crafts reaction between 1-fluoronaphthalene and 4-methylphthalic anhydride, 3-fluoro-6-methyl-1,2-benzanthracene, m.p. 172–173°, has been prepared.

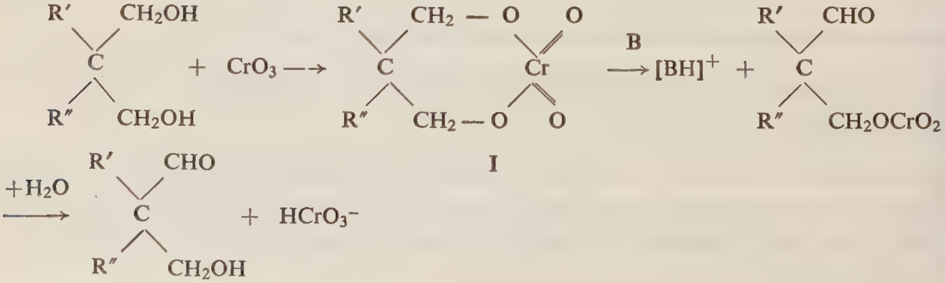
3) In an attempt to utilize the Elbs reaction for the preparation of fluorinated polycyclics (methylcholanthrene, 1,2,5,6-dibenzanthracene), it was observed that whenever the ketone undergoing the Elbs reaction carried the fluorine atom *para* to the carbonyl group, the halogen was eliminated and a halogen-free hydrocarbon formed. Only 4-fluoro-1,2,5,6-dibenzanthracene (m.p. 229°) could be prepared [from (3-fluoro-1-naphthyl)-(1-methyl-2-methyl)-ketone, m.p. 164°].

Details of the preparative procedure and some hypotheses regarding possible reaction mechanisms will be presented.

Chromium trioxide — pyridine oxidation of substituted 1,3-propanediols

Leo A. Pohoryles and Shalom Sarel*, *Department of Pharmaceutical Chemistry, The Hebrew University School of Pharmacy, Jerusalem*

For the preparation of 2,2-disubstituted propanedialdehydes, needed for another research program, the CrO_3 pyridine oxidation of a series of appropriated diols was contemplated. We found that, untilke unsubstituted aiphatic 1,2-, 1,3- and 1,4- diols, and dialdehydes, the oxidation of a series of 2,2-disubstituted propanediols provided almost invariably the corresponding 3-hydroxy-2,2-disubstituted propionaldehydes, isolated as their DNPH. This observation prompted the investigation of the mechanism of oxidation of glycols by means of that reagent and the results obtained are explicable in terms of formation of an intermediate of 1,3-cyclic chromate (I), as depicted below:



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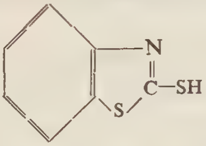
* Lecturer

Use of S^{35} in syntheses and exchange reactions

MICHAEL BENTOV, *Israel Institute for Biological Research, Ness Ziona*

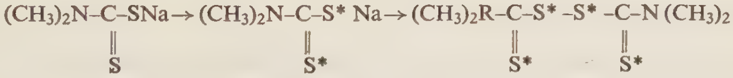
The reactivity of hetero atoms in organic compounds can easily be determined by the use of the radioactive isotopes of these atoms.

When 2-mercapto benzothiazole, a compound used in many industrial processes, is prepared from phenyl isothiocyanate and labelled sulphur, *both* sulphur atoms in the product



are labelled. A product labelled only in the sulphydryl group was prepared by interaction between 2-chlorobenzothiazole and sodium hydrosulphide-S^{35}. A 79% radioactive yield was obtained.

An easy method for the introduction of radioactive sulphur into organic sulphur compounds is the exchange between these compounds and labelled S^{35}. Thus, sodium N–dimethyldithiocarbamate, labelled in both sulphur atoms, was prepared; its oxidation yielded labelled tetramethylthiuram disulphide with a total radioactive yield of 82%.



$$(CH_3)_2N-\underset{\underset{S}{\|}}{C}-SNa \rightarrow (CH_3)_2N-\underset{\underset{S^*}{\|}}{C}-S^*\,Na \rightarrow (CH_3)_2R-\underset{\underset{S^*}{\|}}{C}-S^*-S^*-\underset{\underset{S^*}{\|}}{C}-N(CH_3)_2$$

The analogous exchange reaction between thiourea and S^{35} proceeded with 90% radioactive yield.

SECTION B

BIOCHEMISTRY

First Session Chairman: A. BONDI

Biosynthesis of the sphingolipids

SHIMON GATT, *Department of Biochemistry, The Hebrew University–Hadassah Medical School, Jerusalem*

The metabolism of 1-C^{14} lignoceric acid, the straight chain C_{24} fatty acid, when injected intravenously into rats has been described[1]. No incorporation of lignoceric acid into the brain lipids could, however, be detected in those experiments. Due to the important role of the C_{24} fatty acids as major constituents of the fatty acid portion of the sphingolipids, the metabolic utilization of 1–C^{14} lignoceric acid and other C^{14}-labelled fatty acids in the intact brain has now been studied by intracerebral injection into 14–16 day old rats. The brain lipids were extracted, fractionated on alumina, florisil and silic acid columns and the sphingolipids isolated.

In the intact brain, 1–C^{14} lignoceric acid was not oxidized to $C^{14}O_2$. It was, however, slowly incorporated into the sphingolipids, the peak incorporation resulting 24 hours after injection.

5% of the injected C^{14}-activity of 1-C^{14} acetate was recovered in the brain lipids, the cerebrosides having about 10% of the lipid C^{14}-activity. 60% of the activity cerebrosides resided in the fatty acid portion of the cerebroside molecule.

15% of the injected C^{14}-activity of 1-C^{14} palmitate was found in the brain lipids, the cerebrosides having 1.5% of the lipid C^{14}-activity. Paper chromatography of the fatty acid portion of the cerebrosides showed that most of the C^{14}-activity was incorporated as intact palmitic acid.

The relation of these results to the possible biosynthetic pathways of the sphingolipids will be discussed.

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Biosynthesis of lipids in insects

Alisa Tietz – Devir, *Israel Institute for Biological Research, Ness-Ziona*

The biosynthesis of lipids in two different insects was investigated: (1) Third instar larvae of the housefly (*Musca vicina* Macq.) and (2) Adult locusts (*Locusta migratoria*).

Homogenates were prepared from eviscerated larvae in isotonic KCl — potassium phosphate buffer, *p*H 7.15, and centrifuged for 30 min. at 15,000 g. The clear supernatant incorporated (1-C^{14}) acetate into fatty acids in the presence of Mg^{++} or Mn^{++}, adenosine triphosphate, coenzyme A, glutathione, citrate (or isocitrate) and bicarbonate. Diphospho- and triphosphopyridine nucleotides were routinely added although their effect could not be demonstrated. Malonate slightly but consistently stimulated the system and was therefore also added. Under optimal conditions, approximately 0.16 μ moles of acetate were incorporated into fatty acids per mg. of protein during 2 hours of incubation at 30°. 75% of the synthesized fatty acids was recovered as triglycerides and 25% as phospholipids.

A second system was obtained from the fat body of adult locusts. Homogenates were prepared in isotonic KCl — potassium phosphate buffer, *p*H 7.4, and centrifuged for 5 min at 400 g. The homogenates incorporated (1-C^{14}) acetate into fatty acids in the presence of Mg^{++}, adenosine triphosphate, coenzyme A, glutathione, triphosphopyridine nucleotide, malonate and bicarbonate. α-Ketoglutarate further stimulated the system and was therefore also added. Under optimal conditions approximately 0.18 μ moles of acetate were incorporated into fatty acids per mg of protein during 2 hours of incubation at 30°.

Fatty acid esterification in adipose tissue

G. Rose, *Department of Biochemistry, The Hebrew University–Hadassah Medical School, Jerusalem*

Adipose tissue, since it is predominantly concerned with the synthesis and breakdown of fat, provides an ideal medium for the study of the formation of fatty acid glycerides. In previous studies we were able to show that 1-C^{14} labelled stearic or palmitic acid was rapidly absorbed into rat mesenteric adipose tissue in vitro and could later be recovered as stearic or palmitic triglyceride[1,2]. Esterification was found to be highest in adipose tissue derived from rats previously starved for several days and re-fed on a carbohydrate rich diet 24–48 hours prior to the experiment. The present investigation was carried out to elucidate the enzymatic mechanism of fatty acid esterification and to study the possible precursors of the glycerol moiety of the triglyceride ester.

Adipose tissue of rats in the nutritional state described above was homogenized with a KCl — phosphate buffer mixture and separated into a particular and a soluble fraction. The presence of enzymes participating in fatty acid esterification could be demonstrate-rated as follows: A thiokinase, catalyzing the formation of higher acyl CoA com-pounds, dependent upon CoA, ATP and Mg^{++} was found in the acid particles of the soluble fraction. A condensing enzyme catalyzing the transfer of the acyl group of palmitoyl CoA to α-glycerophosphate, but not to free glycerol, was present in the same acid particle precipitate. Phosphatidic acid phosphatase activity could be demonstrated in the particular fraction, and a Ca^{++} activated lipase was contained in crude adipose tissue homogenate.

Degradation studies carried out on newly *in vitro* synthesized triglycerides in adipose tissue pointed to a new synthesis of all three fatty acid ester bonds.

A scheme for the pathway of fatty acid esterification in adipose tissue is presented and discussed.

REFERENCES

1. Shapiro, B., Chowers, I. and Rose, G., *Proceedings of the 2nd International Conference on the Biochemical Problems of Lipids*, p. 347
2. Shapiro, B., Chowers, I. and Rose, G., 1957, *Biochim. Biophys. Acta*, 23, 115.

Release of glycerol during mobilization of unesterified fatty acids (UFA) from adipose tissue

E. SHAFRIR, *Department of Biochemistry, The Hebrew University–Hadassah Medical School, Jerusalem*

Rat adipose tissue, when stimulated *in vitro* or when removed from the fasting animal, was shown recently to release its triglyceride fatty acid reserves in the form of UFA[1,2]. No concomitant increase in the lipolytic activity could, however, be demonstrated in such tissue. As the release of UFA must involve cleavage of the tissue glyceride esters, the albumin medium serving as an acceptor for UFA was investigated for the presence of glycerol. Glycerol was expected to diffuse readily out of the tissue, since it is only sparingly utilized in adipose tissue[3]. A substance which yielded formaldehyde on oxidation with periodic acid was shown to be released from epinephrine stimulated tissue pads and was identified as glycerol by the isotope dilution method after elution with benzene-butanol 1:3 from a Celite column. About 1 μMole of glycerol was released for every μMole of UFA instead of the expected 1/3 μMole. Glycerol was also released from non-stimulated tissues from fed animals, which do not mobilize fatty acids. These findings along with those reported in the preceding communication suggest that the release of fatty acids by the tissue may be the result of a disturbance in the rate of esterification within the tissue.

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Fatty acid esterification in adipose tissue in relation to fat mobilization

S. KERPEL, E. SHAFRIR AND B. SHAPIRO, *Department of Biochemistry, The Hebrew University–Hadassah Medical School, Jerusalem*

The absorption and esterification of labelled unesterified fatty acids (UFA) from an albumin solution has been demonstrated to occur readily in rat adipose tissue *in vitro*[1]. In the present study the effect of some hormones known to induce mobilization of depot glyceride fat as UFA was studied in a similar system, using 1-C^{14}- palmitate as representative of the UFA group. The decrease in the specific activity of the albumin medium and the increase in the activity of tissue UFA and glyceride pools were measured after 1 hour of incubation at 37°C. In the presence of epinephrine and norepinephrine, the incorporation of palmitate into the tissue triglycerides was strongly inhibited while the tissue UFA pool rose markedly both in quantity and specific activity. The inhibition of esterification is reduced or reversed when glucose is included in the incubation medium. On the basis of these results it is postulated that the UFA mobilizing action of epinephrine may be exerted through a shift in carbohydrate metabolism of the adipose tissue, which limits the availability of a glucose-derived acceptor, necessary for the re-esterification of the tissue fatty acids.

REFERENCE

1. Shapiro, B., Chowers, I. and Rose, G., 1957, *Biochim. Biophys. Acta*, **23**, 115.

Second Session Chairman: L. Bichowsky — Slomnicki

The mechanism of galactose absorption in the small intestine

Ruth Tzur and B. Shapiro, *Department of Biochemistry, The Hebrew University–Hadassah Medical School, Jerusalem*

Galactose absorption from the rat intestine was studied in the intact animal, in the perfused intestine and in the isolated intestinal loop. In the intact animal an average porto-arterial difference of 16.1 mg % galactose was found. At the same time time there was no porto-arterial difference in the total reducing sugar.

In the perfused rat intestine, the entire amount of galactose removed from the intestinal lumen could be recovered in the mucosa and the perfusion fluid. In this case too, no additional reducing sugar appeared in the portal blood. The galactose replaced an equivalent amount of glucose. The presence of glucose in the perfusion fluid was an important factor in galactose absorption, its omission decreasing galactose transport into the blood by 40–50%.

Glucose also accelerated galactose transport in the isolated intestine when added to the outer solution, but caused inhibition when introduced into the lumen. Acetate, succinate and pyruvate, up to the concentration of 50 μM/ml were without effect. Higher concentrations of pyruvate accelerated galactose transport to the same extent irrespective of the site of application.

It is suggested that resorption of galactose is based on an exchange between the galactose in the intestinal mucosa and blood glucose. According to this hypothesis galactose and similar sugars are absorbed into the blood as they are able to combine with a carrier in the intestinal mucosa. The replacement from this carrier and their introduction into the blood depends on the exchange with glucose.

Equilibrium constants in transfructosylation reactions by levansucrase from *Aerobacter levanicum*

Ithak Ohad and Shlomo Hestrin, *Department of Biological Chemistry, The Hebrew University of Jerusalem*

Reversible fructosyl transfer from fructosylaldosides to the anomeric carbon of aldoses, in the presence of levansucrase from *Aerobacter levanicum*, has been described (Hestrin and Avigad, 1958).

Equilibrium constants of this transfructosylation have now been measured for the following systems:

(a) transfer from sucrose to several reducing disaccharides (maltose, melibiose and cellobiose);

(b) transfer from raffinose to pentoses conforming in configuration at C–2 and C–3 to L-threose (D-xylose) and D-threose (L-xylose and D-arabinose).

Production of C^{14}-raffinose with the aid of this transfer reaction is described.

Values of equilibrium constants (*p*H 5.4, 37°C) of this transfer reaction ranged from 0.3–1.9 for the different tested systems.

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1. Hestrin, S. and Avigad, G., 1958, *Biochem. J.*, **69**, 388.

Enzymatic synthesis of α-glucosyl 2-deoxyglucose, an analog of maltose

Zvi Selinger and Michael Schramm, *Department of Biological Chemistry, The Hebrew University of Jerusalem*

As shown by Fitting and Doudoroff, maltose is formed from β-glucose-1-phosphate and glucose in a reversible reaction catalysed by maltose phosphorylase. It was also demonstrated that glucose can be replaced by xylose to form α-1,4 glucosyl xylose[1]. The possibility that other sugars or polyalcohols might replace glucose to form analogs of maltose was tested. 2-deoxyglucose reacted with β-glucose-1-phosphate to yield α-glucosyl 2-deoxyglucose.

REFERENCE

1. Fitting, C. and Doudoroff, M., 1952, *J. Biol. Chem.*, **199**, 153.

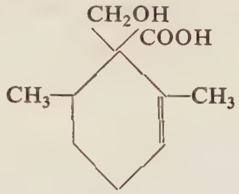
Oleuropein, the bitter principle of olives

B. Shasha and J. Leibowitz, *The Hebrew University of Jerusalem*

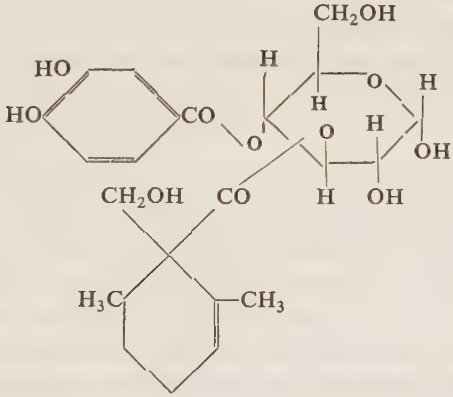
This natural product is defined in the botanical and chemical literature as a glucoside whose structure is similar to that of tannin. Its aglycon is described as being optically inactive and having caffeic acid as a component part.

We have found that the oleuropein, as prepared in previous researches is not a homogeneous compound. The purified compound ($C_{23}H_{30}O_{11}$) is not a glucoside but a double ester of glucose with two organic acids, protocatchuic acid and oleuropeic acid. The latter is optically active and has not been reported in the previous literature.

The structure of the oleuropeic acid has been proved by hydrogenation, dehydrogenation, ozonolisation and decomposition by acid to be:



These acids are linked to glucose at C 3 and C 4. The structure of oleuropein has been established as:



Conversion of β-glucose-1-phosphate to glucose-6-phosphate by a specific mutase

RUTH BEN-ZVI AND MICHAEL SCHRAMM, *Department of Biological Chemistry, The Hebrew University of Jerusalem*

Extracts of *Neisseria meningitidis* and *N. perflava* are known to catalyse formation of β-glucose-1-phosphate by phosphorolysis of maltose[1]. The preparations have now been found to contain an enzyme which further converts β-glucose-1-phosphate to glucose-6-phosphate in the presence of Mn^{++} or Mg^{++}.

The enzyme has been purified 90 fold from *N. perflava*. Studies with the purified preparation reveal that the conversion of β-glucose-1-phosphate to glucose-6-phosphate is reversible and that α-glucose-1-phosphate is not a substrate for this enzyme.

REFERENCE

1. FITTING, C. AND DOUDOROFF, M., 1952, *J. biol. Chem.*, **199**, 153.

Synthesis of cellulose by *A. xylinum* cells from citric acid cycle intermediates

Z. Gromet-Elhanan, *Department of Biological Chemistry, The Hebrew University of Jerusalem*

Resting cells prepared from sugar-grown *A. xylinum* cultures synthesized cellulose from sugars and from substrates known to be metabolized via the pentose cycle[1]. Together with results obtained from experiments with specifically labeled hexoses, these findings suggested that cellulose arises in *A. xylinum*, as in green plants, from an intracellular pool of hexose phosphate formed by way of a pentose cycle[2].

In a study on oxidative pathways in sugar-grown *A. xylinum* cells, organic acids were found to be oxidized by way of a citric acid cycle which participates also in sugar oxidations. But cellulose synthesis from citric acid cycle intermediates was never encountered in these cells. It is assumed that ways enabling flow of material from pentose cycle to citric acid cycle are present in sugar-grown *A. xylinum* cells, but flow of material in the opposite direction is impossible.

On growing *A. xylinum* on citric acid cycle intermediates, cells were obtained which mediated cellulose synthesis from these intermediates as well as from sugars. Growth was indicated by formation of a pellicle which was identified as cellulose similar to that formed by the sugar-grown cells. Resting cells prepared from cultures grown on citric acid cycle intermediates were able, under appropriate conditions, to synthesize cellulose from these intermediates. Synthesis of cellulose from organic acids indicates that in these cells the connection between pentose cycle and citric acid cycle works in both directions.

Possible ways leading from the citric acid cycle to the pentose cycle will be discussed.

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2. Schramm, M., Gromet, Z. and Hestrin, S., 1957, *Nature*, **179**, 28.

Third Session Chairman: S. Hestrin

The behavior of 6-hydrazinopurine towards mammalian xanthine oxidase (XO)

H. Kwietny,* F. Bergmann and H. Ungar, *Department of Pharmacology, The Hebrew University–Hadassah Medical School, Jerusalem*

6-Hydrazinopurine (I) is attacked by XO along the same pathway as adenine (i.e. I — II — III):

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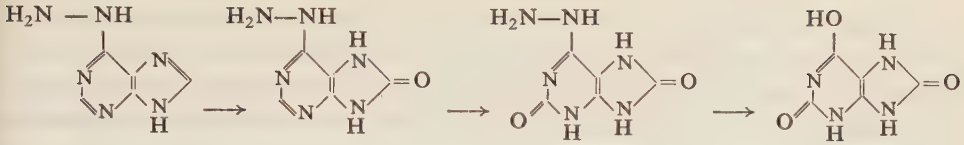
* Lecturer



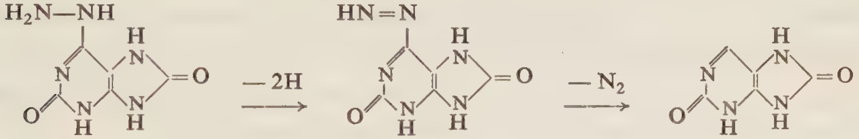
I II III IV

However, 2,8-dihydroxy-6-hydrazinopurine (III) is not stable under the experimental conditions and is degraded further to uric acid (IV). Conversion of III to IV takes place only in the presence of XO.

IV is not formed by enzymatic "hydrolysis" of III, because no trace of hydrazine was found in the final solution.

Likewise, III is not split reductively at the N-N bond, because the resulting 2,8-dihydroxyadenine is stable towards enzymatic attack and does not yield uric acid.

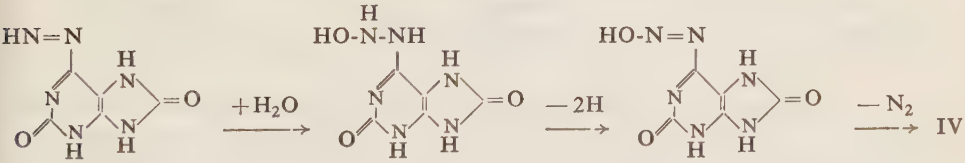
It appears possible that III is dehydrogenated to a substituted di-imide RN = NH, which splits off N_2 to give 2,8-dihydroxypurine (V):



III Di-imide V

However, no indication for the formation of (V) as intermediate has been obtained.

It is assumed that the diazonium derivative VI is formed by double dehydrogenation, because phenylhydrazine is also oxidized enzymatically to phenol:



Di-imide VI

A D-lactic dehydrogenase from *Leuconostoc mesenteroides*

E. KAUFMANN* AND S. DIKSTEIN, *Israel Institute for Biological Research, Ness-Ziona*

Leuconostoc mesenteroides contains a lactic dehydrogenase stereospecific for the D-form.

Enzymatic activity was assayed by the reduction of 2,6-dichlorophenol indophenol. Under aerobic conditions the pH optimum was found to be 6.5. K_m for D-lactate in phosphate buffer is 1.5×10^{-5}M. Ferricyanide, cytochrome or molecular oxygen could not serve as hydrogen acceptors. The enzyme is DPN – independent. Substitution at the β-carbon causes a sharp decrease in activity. Other α- and β-hydroxy acids tested were not attacked, nor were glycolic acid and the ethyl ester and amide of DL-lactic acid. This points to a very restricted substrate structure of $CH_3CHOH\text{-}COO^-$.

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* Lecturer

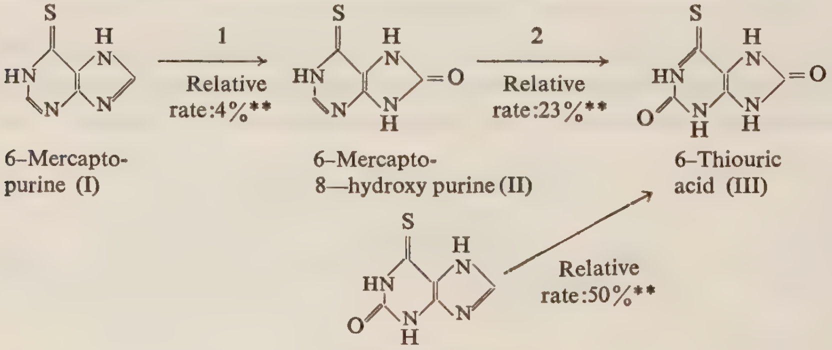
Atebrin and p-chloromercuribenzoate do not inactivate the enzyme. Oxalate and fluoropyruvate are powerful inhibitors. Amongst the bivalent cations, Hg^{++} and Cu^{++} show strong inhibitory action, which can be reversed by histidine. This suggests the presence of an imidazole group at the active surface of the enzyme, an assumption further strengthened by the irreversible inhibitory action of iodine.

Substrate and inhibitor specificity indicate that the lactic dehydrogenase of *L. mesenteroides* differs from the known ones of muscle and yeast.

The enzymatic oxidation of 6-mercaptopurine

F. Bergmann and H. Ungar*, *The Department of Pharmacology, The Hebrew University–Hadassah Medical School, Jerusalem*

6-Mercaptopurine (I), the sulphur analog of hypoxanthine, is oxidized by mammalian xanthine oxidase (XO) first at carbon atom 8 and then at C-2:



6–Mercapto-purine (I)

6–Mercapto-8—hydroxy purine (II)

6–Thiouric acid (III)

6–Thioxanthine (IV)

** All rates are compared with that of xanthine

Because step 1 is considerably slower than the subsequent oxidation of II, the latter does not accumulate sufficiently to be identified on a paper chromatogram, when the reaction is interrupted in the middle. However, the formation of 6-mercapto-8-hydroxypurine (II) as intermediate and the complete absence of 6-thioxanthine (IV) in the reaction mixture can be demonstrated by the "method of isosbestic points". At 308 mμ, the isosbestic point of I and II, no change can be observed during the first 20 min of the reaction, thus providing evidence that IV is not formed. On the other hand, at 240 mμ, the isosbestic point of I and IV, the optical density increases linearly during the initial reaction period. In this way it is possible to measure the rate of the first oxidation step (I → II).

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* Lecturer

The different pathways of enzymatic attack on I and hypoxanthine indicate that the respective ES-complexes must possess different structures, due to the weak tendency of the sulphur atom to form hydrogen bonds. Most important is the observation that the oxidation of I is completely analogous to the oxidation of 4-hydroxypteridine[1].

REFERENCE

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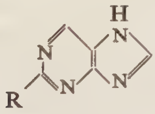
Oxidation of 2-aminopurines by mammalian xanthine oxidase (XO)

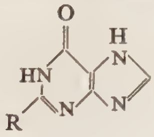
F. Bergmann*, H. Kwietny and H. Ungar, *Department of Pharmacology, The Hebrew University–Hadassah Medical School, Jerusalem*

It has been shown previously that 2-aminopurine is attacked at position 6 to give guanine[1] which is resistant towards enzymatic oxidation.

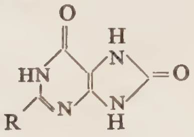
We have now observed that 2-methylaminopurine (Ia) follows the same pathway, but the intermediate IIa is oxidized further in position 8 (III):



I



II

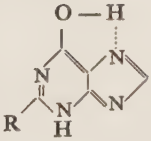


III

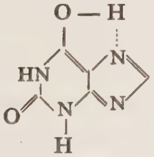
a: R = $NHCH_3$
b: R = $N(CH_2)_3$

The intermediate IIa can easily be identified on a paper chromatogram.

2-Dimethylaminopurine (Ib) is not oxidized by XO, but N^2, N^2-dimethylguanine (IIb) is oxidized even more rapidly than IIa. The pertinent data are collected in Table I. These observations make it probable that IIa and IIb can be tautomerized in the ES-complex to an active form A, corresponding to that of xanthine (B)[2], whereas such a shift cannot be achieved with guanine itself. Possible reasons for this difference will be discussed.



A



B

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* Lecturer

TABLE I

Purine derivative	Relative rate of oxidation (xanthine = 100)	R_F*
1. 2-Aminopurine	17.5	0.56
2-Methylamino-purine	5.4	0.67
2-Dimethylaminopurine	0	0.77
2. Guanine	0	0.4
N^2-Methylguanine	0.3	0.56
N^2, N^2-Dimethylguanine	0.6	0.63

* Descending method. Solvent: 95% ethanol-85 vols., acetic acid-5 vols., water-10 vols.

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The occurrence of a TPNH specific hydroxylamine reductase in enterobacteriaceae and the repression of its formation by cysteine

J. Mager and Miriam Weiss-Zehavi, *Department of Biochemistry, Hebrew University–Hadassah Medical School, Jerusalem*

An enzyme capable of reducing hydroxylamine to ammonia at the expense of TPNH as hydrogen donor has been found in cell-free sonic extracts of *E. coli*, *A. aerogenes* and related species. The enzyme was purified more than a hundredfold by a procedure combining protamine precipitation, ammonium sulphate fractionation and calcium phosphate gel adsorption. The reaction balance studied with the purified preparation showed that the amount of TPNH oxidized was equivalent to the amounts of hydroxylamine which disappeared and ammonia which was produced. Thus the stoichiometry of the reaction is consistent with the following formulation:

$$NH_2OH + TPNH + H^+ \longrightarrow NH_4OH + TPN$$

The K_m for hydroxylamine was found to be 3.9×10^{-3}M. The pH of optimum activity was close to 9. The reaction was virtually irreversible. DPNH failed to replace TPNH in its function as hydrogen donor. Hydrazine and oximes of pyruvate and α-ketoglutorate could not serve as alternate substrates in place of hydroxylamine

The activity of the purified enzyme was enhanced by FAD and inhibited by KCN and SH-reagents.

The enzyme formation by the growing cells was strikingly repressed by addition of peptone or a mixture of amino acids to the minimal culture medium.

The inhibitory amino acid was identified as cysteine which in relatively small amounts (about 0.25 μmole/ml) completely abolished the production of hydroxylamine reductase. Other thiol compounds, metabolically unrelated to cysteine, such as mercaptosuccinate or mercaptoacetate, were without effect on the enzyme yield.

It appears likely that the observed phenomenon represents a negative feedback control mechanism, thus suggesting a possible relationship between the metabolic function of hydroxylamine reductase and the biosynthesis of cysteine. The validity of this hypothesis is being currently examined.

Fourth Session Chairman: J. MAGER

The site and mechanism of ATP formation in photophosphorylation by chloroplasts

M. AVRON, *Department of Biochemistry, The Weizmann Institute of Science, Rehovot*

The type of photophosphorylation by chloroplasts in which oxygen is evolved, TPN is reduced, and ATP is formed, simultaneously, was further investigated. The addition of an inhibitor which acts in the oxygen evolution path, inhibited all three reactions. The subsequent addition of a reducing agent, relieved the inhibition of TPN reduction, but had slight to no effect on the evolution of oxygen or the formation of ATP. The results are interpreted as indicating the site of ATP formation to be coupled to reactions associated with the oxygen evolution path, rather than to those associated with the reduction of added oxidants.

Further evidence, through the study of exchange reactions, supports the previously suggested mechanism for photophosphorylation[1].

REFERENCE

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Some intracellular factors effecting the spontaneous swelling of rat liver mitochondria

Y. AVI-DOR* AND S. H. KINDLER, *Israel Institute for Biological Research, Ness-Ziona*

The swelling of rat liver mitochondria upon dilution in 0.25 M sucrose is preceded by a temporary equilibrium. The length of this phase is dependent on the extent of the dilution *p*H and temperature. During this lag a reaction is presumed to take place which leads to swelling. The calculated energy of activation of such a reaction is compatible with a biological oxidation process.

Following aging of concentrated mitochondrial suspensions at 0°C a substance accumulates which greatly reduces the lag phase of fresh, diluted mitochondria. A substance of very similar properties is present in the microsomal fraction of the liver homogenate upon isolation. Both the mitochondrial and the microsomal "swelling factors" are of a lipid nature. Their effect can be completely suppressed by some commercial antioxidants, e.g. octyl gallate.

The origin and the mechanism of formation of these swelling factors will be discussed.

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* Lecturer

Enzymatic activity of Novikoff tumor mitochondria in relation to isolation procedures

S. H. KINDLER* AND Y. AVI-DOR, *Israel Institute for Biological Research, Ness-Ziona*

The energy-yielding and synthetic processes of tumor mitochondria are less well understood than those of normal tissue. Tumor mitochondria isolated according to standard procedures (isotonic sucrose) may show impaired function or even apparent lack of several enzymatic systems.

The mitochondrial fraction of Novikoff hepatoma was isolated in various media. Using 0.25 M sucrose, no glutamic dehydrogenase or inorganic phosphate (P_i^{32}) ATP exchange could be demonstrated. When the isolation medium was supplemented with several factors, most active among them being serum albumin, these enzymatic activities were restored. Likewise the ATP-ase pattern and osmotic behaviour resembled more closely those of normal liver mitochondria. Thus an indication of an altered permeability barrier was obtained.

The incorporation of P_i^{32} into ATP and the mitochondrial membrance was studied. The nucleotide specificity and the kinetics of the exchange reaction were examined. The latter differed in several respects from that of liver mitochondria. Specific features of mitochondria obtained from tumor will be discussed.

The effect of temperature and 2,4-dinitrophenol on the nucleotide triphosphatase activity of myosin[1]

NATHAN SHARON[2], HARVEY M. LEVY AND D. E. KOSHLAND, Jr., *Biology Department, Brookhaven National Laboratory, Upton, N.Y., U.S.A.*

Many diverse agents, such as 2,4-dinitrophenol (DNP), SH reagents, metal ions, temperature and actin, are known to affect the nucleotide triphosphatase activity of myosin. These effects appear to be complex and have been poorly understood. However, a detailed analysis of the effect of temperature and DNP on the rates of hydrolysis of adenosine triphosphate (ATP), inosine triphosphate (ITP) and tripolyphosphate in presence of various ions has revealed a consistent pattern of action. The behavior of the enzyme under the conditions studied is related to a temperature induced change in the protein conformation with consequent effects on its catalytic properties. That such a change occurs is deduced, for example, from the appearance of "breaks" in the Arrhenius plots for the rates of hydrolysis by myosin of ITP and of ATP in the presence of DNP.

The general implications of these findings to the mechanism of action of the various agents on the nucleotide triphosphatase activity of myosin, as well as to muscular contraction, will be discussed.

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[1] Research carried out at Brookhaven National Laboratory under the auspidces of the U.S. Atomic Energy Commission.

[2] On leave of absence from the Weizmann Institute of Science.

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* Lecturer

Fifth Session Chairman: J. LEIBOVITZ

Complete tryptic hydrolysis of DL-lysine methyl ester

Y. SHALITIN AND Y. WOLMAN, *Department of Biophysics, The Weizmann Institute of Science, Rehovot*

In addition to its proteolytic action trypsin acts also as an esterase, hydrolyzing the esters of L-lysine and L-arginine[1]. In recent years it has been shown that it acts also on esters of ε-amino caproic acid[2]. It seemed, therefore, of interest to examine whether esters of lysine having the D-configuration are split by trypsin.

On analyzing the action of trypsin on methyl ester of DL-lysine by means of the pH-static method, it was found that complete hydrolysis occurred in two stages: The L-isomer was hydrolyzed very rapidly while the D-isomer was hydrolyzed slowly (Table I).

It appears therefore, that trypsin can digest not only the L configuration of the substrate but also its antipodes, although less favourably.

TABLE I

Substrate	k_3* (mole/1/min/mg enzyme/ml)	$K_m \times 10^3$* (mole/1)
L-lysine methyl ester	0.040	3
D-lysine methyl ester	0.001	10
ε-amino methyl caproate	0.0002	0.15

* The hydrolysis constants k_3 and the Michaelis constants K_m ($K_m = \frac{k_3 + k_3}{k_1}$) for the various substrates tested were found at $p\text{H} = 6.0$ in the presence of 0.05 N CaCl_2.

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A trypsin column

ATARA BAR-ELI AND EPHRAIM KATCHALSKI, *Department of Biophysics, The Weizmann Institute of Science, Rehovot*

Water-insoluble derivatives of trypsin with enzymatic activity were prepared by coupling polytyrosyl-trypsin with copolymers of leucine and p-aminophenylalanine. Columns with trypsin activity were prepared from these water-insoluble derivatives.

A water-soluble polytyrosyl-trypsin (PTT) was prepared by using trypsin as initiator for the polymerization of N-carboxy-tyrosine anhydride[1,2]. The tyrosine content of the PTT thus obtained was by 5-10% higher than that of intact trypsin. The proteolytic activity of PTT equalled 75% of that of trypsin.

A water-insoluble trypsin preparation was obtained by coupling PTT with a diazonium salt derived from a copolymer of *p*-aminophenylalanine and leucine. The polytyrosyl-trypsin-azo-copolymer contained trypsin to an extent of 20–25% of its weight. The amount of protein in the insoluble product was derived from the amount of valine formed on exhaustive acid hydrolysis[3].

The enzymatic activity of the final product approached 10–20% of that to be expected from the total amount of protein. Trypsin was found to lose 50% of its activity during the conditions of the coupling reaction.

The activity of the water-insoluble trypsin on arginine methyl ester (AME) and benzoyl-arginine methyl ester was determined by the *p*H-stat titrator, type TTT 1.

A trypsin column was prepared and its activity on various substrates tested. The products obtained were free of protein. The digestion of poly-L-lysine protamine and oxidized insulin[4] could be readily achieved.

The dependence of the extent of hydrolysis on concentration and rate of flow was tested, using AME as substrate.

A trypsin column which was kept for seven months at 2°, in the presence of 0.0025N HCl, retained half of its original activity.

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An intermediate formed in the conversion of pepsinogen to pepsin

Hava Neumann and Nathan Sharon, *Department of Biophysics, Weizmann Institute of Science, Rehovot*

By activation of commercial pepsinogen at *p*H 3, followed by $MgSO_4$ fractionation, "*p*H 3 pepsin" was obtained. The enzymatic properties of this "*p*H 3 pepsin" were investigated and compared with those of pepsin prepared by the activation of pepsinogen at *p*H 2 ("*p*H 2 pepsin")[1], and with those of commercial crystalline pepsin.

The "*p*H 3 pepsin" showed enzymatic activity toward hemoglobin[2] equal to that of "*p*H 2 pepsin" or crystalline pepsin. However, it differed from the latter in the following respects: (1) Its hydrolytic activity towards N-carbobenzoxy-L-tyrosyl-L-phenylalanine was several times greater, and (b) it catalysed the hydrolysis of the substrates N-carbobenzoxy-L-phenylalanyl-L-tyrosine, N-carbobenzoxy-L-tyrosyl-L-tyrosine, N-carbobenzoxy-L-glutamyl-L-tyrosine and N-*p*-aminobenzoyl-L-tyrosyl-L-tyrosine similarly to the "pH 2 pepsin" or crystalline pepsin, but in contradistinction to "pH 2 pepsin" or crystalline pepsin, no transpeptidation reaction occured on the incubation of the above substrates with the "pH 3 pepsin".

The "pH 3 pepsin" is unable to perform the amine transfer transpeptidation leading to the formation of oligopeptides with a number of amino acid residues greater than in the substrates.

"pH 3 pepsin" could readily be converted to "pH 2 pepsin" by lowering the pH to 2; it is quite likely that "pH 3 pepsin" is an intermediate formed in the usual activation process of pepsinogen. This conversion seems to be irreversible. Attempts are being made to detect peptides liberated during the conversion of "pH 3 pepsin" to "pH 2 pepsin".

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Proteolytic activity in egg white

H. Neumann and M. Sela, *Department of Biophysics, The Weizmann of Science, Rehovot*

Several workers [1-4] have searched for proteolytic activity in egg white, but it has never been unequivocally established. The development of new selective techniques for the detection of the proteolysis products, as well as the use of synthetic polypeptides as potential substrates, enabled us to demonstrate proteolytic activity in the egg white of hens.

In most experiments 0.5 mg substrate (in 0.1 ml solution buffered at pH 7.8) was incubated, at 37°, with the egg white (1 ml, obtained after blending the whites of twenty fresh eggs in a Waring Blender). After 24 hours the proteins in the reaction mixture were precipitated with 5% trichloroacetic acid, and the supernatant fluid was analysed for the enzymatic products.

The following substrates were digested under the above conditions to an extent of more than 30 per cent; poly-L-proline, poly-L-hydroxyproline, copolymers of L-tyrosine with DL-alanine or L-glutamic acid, natural poly-γ-glutamic acid and DL-alanyl-DL-alanine. Proline[5] and hydroxyproline[6] were followed colorimetrically Tyrosine was located on chromatograms with diazotized sulphanilic acid and quantitated with ninhydrin. The other free amino acids formed by proteolysis were analysed by chromatography, followed by ninhydrin colorimetry[7].

Among the materials that were not significantly attacked by the egg white, and gave results similar to blank experiments, were: poly-L-lysine, poly-L-α-glutamic acid, poly-L-aspartic acid, a copolymer of L-tyrosine and L-lysine, and a copolymer of L-aspartic acid and L-lysine.

No proteolytic activity was observed when the reaction mixtures were heated at zero time to 60° and incubated at 37° for 24 hours. No bacterial contamination was found in the egg white within 48 hours of incubation under the experimental conditions used, as examined by growth on agar slants or in nutrient broths. It may thus be concluded that the proteolytic activity observed is of an enzymatic character and is present in the uncontaminated egg white.

It is not yet clear if one or more enzymes are responsible for the proteolytic activity described. Fractionation experiments[8] were made in order to separate the different enzymatic activities as well as to concentrate them in definite fractions. We found that the conalbumin-globulin fraction contained almost all the activity on poly-L-proline, but no activity on poly-L-hydroxyproline.

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$\alpha \rightarrow \gamma$ Shift and optical rotation of penicillinase in guanidine HCl

N. Citri, N. Garber and M. Sela, *Department of Bacteriology, The Hebrew University–Hadassah Medical School, Jerusalem, and Department of Biophysics, The Weizmann Institute of Science, Rehovot*

Exopenicillinase of *Bacillus cereus* can exist in two antigenically different states, which also differ in their reactivity towards iodine[1]. The transition from the "iodine-resistant" α-state to the "iodine-sensitive" γ-state ($\alpha \rightarrow \gamma$ shift) does not seem to affect the enzymic activity of penicillinase[2]. It can be brought about through adsorption onto a charged surface or through exposure to alkali[1] or urea[3]. The effect of varying concentrations of another hydrogen-bond breaking agent, guanidine hydrochloride, has now been studied.

At low concentrations of guanidine-HCl the $\alpha \rightarrow \gamma$ shift is inverted. This paradoxical behaviour can be explained by an electrostatic effect shared by other electrolytes. Thus electrolytes in general were shown to prevent the $\alpha \rightarrow \gamma$ shift or to cause a $\gamma \rightarrow \alpha$ shift.

At progressively higher concentrations of guanidine-HCl the sequence of changes is:

$$\alpha \rightleftharpoons \gamma \text{ rev.} \longrightarrow \gamma \text{ irrev.} \rightleftharpoons \text{inactive (rev.)} \longrightarrow \text{inactive (irrev.)}$$

The above observations lend support to the assumption (1) that the $\alpha \rightarrow \gamma$ shift as measured by change in sensitivity to iodine (and wherever feasible by change in the antigenic properties) reflects a partial unfolding of the molecule. A direct confirmation of such unfolding has now been obtained by correlating the $\alpha \rightarrow \gamma$ shift with a change in the optical rotatory properties of penicillinase.

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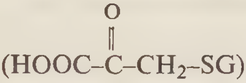
A study of the effect of particle-bound γ-glutamyltranspeptidase on the product of interaction of fluoropyruvate with glutathione

Y. Avi-Dor, *Israel Institute for Biological Research, Ness-Ziona*

"Glutathione-pyruvate" ( $HOOC-\overset{\overset{O}{\|}}{C}-CH_2-SG$), a product of the interaction glutatione (GSH) with fluoropyruvic acid, is hydrolysed by an enzyme present in rat kidney homogenates. This enzyme is bound to microsomal particles. The enzymatic reaction could be followed spectrophotometrically. The products of the reaction were identified by paper chromatography, paper electrophoresis and by spectrophotometrical methods. Glutamate and "cysteinylglycine-pyruvate" are formed in the absence of added amino acids while transpeptidation takes place when a suitable acceptor is present. The identity of the microsome-bound enzyme with γ-glutamyltranspeptidase is thus strongly suggested .Some quantitative aspects of this enzyme activity have been studied using the spectrophotometrical method.

A *p*H-static method for the assay of arginase

Meir Rigbi, *Department of Biological Chemistry, The Hebrew University of Jerusalem*

Arginase activity has been assayed by titration with alkali by Edlbacher[1] using Sorensen's formol titration method, and by Linderstrom-Lang, Weil and Holter[2] using an alcohol-acetone mixture. The present communication describes a rapid method for the assay of arginase, which does not involve the withdrawal of samples, by the rate at which alkali must be added to arginine-arginase systems in order to maintain a *p*H of 10.2. All three methods are based on the fact that the ornithine formed has a less basic δ-amino group than the guanidino group of arginine.

Before assay, the enzyme preparation is activated by incubation at 37° for 3 to 4 hours in the presence of 0.05 M $MnSO_4$ and 0.05 M maleate at *p*H 7, as described by Greenberg[3]. It is then diluted in water, 1:5 or more, so that 0.5 ml. of the diluted solution when added to 4.5 ml. of a 0.316 M solution of L-arginine at *p*H 10.2, will require the addition of at least 0.5 micromole of alkali per minute (5 μl of 0.1 N NaOH).

4.5 ml. of 0.316 M L-arginine hydrochloride at a pH slightly under 10.2 (about 90 percent of an equivalent amount of alkali are required) are now introduced into a water-jacketed reaction-vessel maintained at 25° and connected to the pH-stat and recorder. The reaction-vessel is provided with a magnetic stirrer and fitted with a rubber stopper through which pass the glass electrode, agar bridge, delivery tube of the Agla micrometer syringe, inlet and outlet tubes for the passage of nitrogen. The pH is adjusted to 10.2 and 0.5 ml. of activated enzyme is introduced, thus bringing the arginine concentration to 0.285 M. The addition of alkali vs. time (using 0.1 N to 0.5 N NaOH) is recorded for the first ten minutes of the reaction, arginase activity being expressed as the number of micromoles of alkali added per minute to a 0.285 M solution of L-arginine at pH 10.2 and 25° per unit quantity of the enzyme.

Each micromole of alkali added at 25° and pH 10.2 is equivalent to 2.5 micromoles of L-arginine hydrolysed, and the method described tallies well with that described by Greenberg[3], based on the colorimetric estimation of urea as dixanthyl urea. However, the method is not suitable for use at pH 9.5, as at this pH the large buffer capacity of arginine greatly reduces its sensitivity.

The ratio of alkali added to arginine is in good agreement with that calculated from a comparison of the titration curve of arginine alone and with that of a mixture of equivalent amounts of ornithine and urea. When powerful arginase preparations are used, the total amount of alkali added ,up to the completion of the reaction, may also serve as a means for the determination of small quantities of L-arginine in solution (10 to 200 micromoles).

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Purification of C-esterase

F. Bergmann and S. Rimon*, *The Department of Pharmacology, The Hebrew University–Hadassah Medical School, Jerusalem*

C-esterase[1] can be separated from B-esterase and DFPase, present in hog's kidney extract, by passage through a DEAE cellulose column. The pure enzyme does not any longer contain a dialysable cofactor and is not activated by p-chloromercuribenzoate (CMB). However, in a certain group of the higher fractions, which are devoid of C-esterase activity, the latter appears after incubation with CMB. This indicates the presence of hidden enzyme, bound to an inhibitor. Addition of these fractions to pure C-esterase does *not* cause inhibition. This shows that the special group of "inactive", but activable fractions contains a *stoichiometric* compound "C-esterase + native inhibitor", which is adsorbed on the column more strongly than the free enzyme and thus can be separated from it. This indicates a rather high molecular weight of the native inhibitor, whose activity is probably due to an SH-group.

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* Lecturer

DFPase, which belongs to the group of sulphydryl enzymes[2], is clearly differentiated from the inhibitor.

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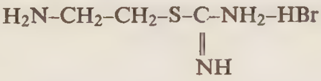
Sixth Session Chairman: Y. Avi-Dor

Derivatives of β-mercaptoethylamines as protecting agents against radiation

A. Kaluszyner* and E. D. Bergmann, *Israel Institute for Biological Research, Ness-Ziona*

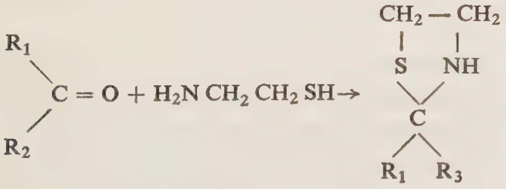
The problem of chemical protection against radiation, i.e. of the use of chemical compounds in order to raise the permissible level of ionising radiation, has attracted much attention in recent years. The known protective agents are the β-mercaptoethylamines and the aminoalkyl-isothiouronium salts



$$H_2N{-}CH_2{-}CH_2{-}S{-}\underset{\underset{NH}{\|}}{C}{-}NH_2{-}HBr$$

(or the isomeric mercaptoalkyl-guanidines formed from them in the first stage).

We have prepared a series of *thiazolidines* by condensation of carbonyl compounds with various mercaptoamines, e.g.



Some of the compounds, especially 2-hexamethyleno-thiazolidine and 2,2,4,5-tetramethyl-thiazolidine have shown outstanding biological activity.

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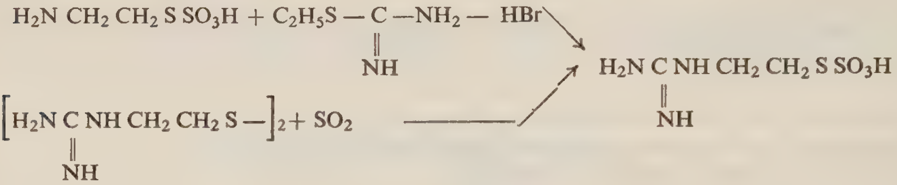
* Lecturer

A number of *aminoalkyl-thiosulphuric acids* ("Bunte salts") have been prepared from bromoalkylamines and sodium thiosulphate, e.g.

$$BrCH_2CH_2NH_2HBr + Na_2S_2O_3 \longrightarrow H_2N\,CH_2CH_2S\,SO_3H$$

Finally, *β-guanidoethyl-thiosulphuric acid* was prepared in two ways:



$$H_2N\,CH_2\,CH_2\,S\,SO_3H + C_2H_5S-\underset{\underset{NH}{\|}}{C}-NH_2-HBr \searrow$$

$$\nearrow H_2N\,\underset{\underset{NH}{\|}}{C}\,NH\,CH_2\,CH_2\,S\,SO_3H$$

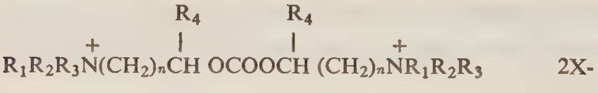
$$\left[H_2N\,\underset{\underset{NH}{\|}}{C}\,NH\,CH_2\,CH_2\,S-\right]_2 + SO_2 \longrightarrow$$

The lecture will also present some pharmacological and toxicological data.

Bis-trialkylammoniumalkanol esters of carbonic acid. A new series of compounds showing curare-like activity

LEO A. POHORYLES* AND SHALOM SAREL, *Department of Pharmaceutical Chemistry, The Hebrew University School of Pharmacy*

In view of the existing knowledge regarding the relationship between structure and curarizing activity of quaternary ammonium salts, we contemplated the synthesis of a new series of bis-trialkylammoniumalkanol carbonates (I) to determine their curarizing action. This study was undertaken in view of the great stability shown by open-chain carbonates towards hydrolysis both in acid and alkaline solutions.



$$R_1R_2R_3\overset{+}{N}(CH_2)_n\overset{\overset{R_4}{|}}{CH}\,OCOO\overset{\overset{R_4}{|}}{CH}\,(CH_2)_n\overset{+}{N}R_1R_2R_3 \qquad 2X^-$$

(I)

The synthetical method used in this study involved 1) the phosgenation of an appropriate ω-dialkylaminoalkanol, followed by 2) quaternization of the corresponding base by means of alkyl halide. Preliminary pharmacological studies, performed by Dr. L. Wislicky of the Department of Pharmacology, The Hebrew University-Hadassah Medical School, indicate that maximum of curarizing activity is obtained when $R_1 = R_2 = R_3 = CH_3$; $R_4 = H$; $n = 3$. The structure-activity relationship in this series will be discussed.

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* Lecturer

A study on the toxicity and the biochemical effects of mono-fluoromalonate

A. CHARI-BITRON, *Israel Institute for Biological Research, Ness-Ziona*

Mono-fluoromalonate (FM), a recently synthesized[1] fluoro-substituted metabolite, was found to be toxic to guinea-pigs, mice and rats. Administration of FM *in vivo* caused convulsions, rise in blood sugar, and accumulation of citrate in the different tissues examined, the last effect being most pronounced in the kidneys.

In vitro, using guinea-pig kidney particles as enzyme source and fumarate as substrate, FM caused an accumulation of citrate and an inhibition of oxygen uptake.

Both *in vivo* and *in vitro*, addition of acetate or malonate decreased the citrate accumulation.

Comparison of the effects of FM with those of fluoroacetate (FA) showed a marked similarity. This fact gave rise to the suggestion that the toxicity of FM could be a result of its prior conversion to FA or fluoroacetyl-CoA.

The inhibitory action of FM on succinic dehydrogenase and on oxaloacetic decarboxylase was also studied. FM proved to be 10–15 times less effective than the powerful inhibitor malonate.

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On the mechanism of resistance of *Micrococcus pyogenes* (var. aureus) to basic polypeptides

LEAH BICHOWSKY-SLOMNICKI, *Department of Biophysics, The Weizmann Institute of Science, Rehovot*

It was previously shown[1] that basic poly-L-amino acid copolymers composed of ornithine and leucine (OL) and ornithine leucine and alanine (OLA) are surface active agents and that their action on bacteria is mainly bactericidal.

The present report deals with the properties of *M. pyogenes* strains, which became resistant to the above mentioned polymers. Resistant strains were obtained by repeated transfers of single colony cultures in liquid casein hydrolysate-salt-glucose medium containing increasing concentrations of the drug. In this way a thousandfold increase to drug resistance was obtained. In most cases the strains remain resistant after many transfers (about 70) in drug free medium. These strains also showed a partial resistance towards natural basic peptide antibiotics such as gramicidin "S", thyrocidine and polymyxine. They remain sensitive to polyornithine, polylysine and many other antibiotics.

The growth curves of strains highly resistant to OL have been studied in the presence of increasing concentrations of the drug. It was found that in the concentration range of 2.5 to 25 μg/ml there is a stready decrease in growth rate of the bacteria with increase of concentration of the drug. But on further increase of the concentration from 25–50 μg/ml to 500 μg/ml an increase of the growth rate of the same strains with increase of concentration was noted.

In strains resistant to up to 25 μg/ml of the drug no detectable change in the surface charge could be noted. But in the highly resistant strains the surface charge which is normally negative changed to a positive one at *p*H 6.3, the standard *p*H of the measurements. The magnitude of the charge increased with the increase in concentration of the drug. The study of the *p*H dependence curves of these cells reveals that up to *p*H 6.8–7.0 the cells are positively charged and they became negative with increasing *p*H. A basic substance possibly related to the cell envelope could be separated from the positively charged cells by shaking in M/15 phosphate buffer at *p*H 8 for 18 hours in the cold. After this treatment the cells were negatively charged. The properties of this extracted substance are under study.

The resistant strains grown in the absence of the drug lose the ability to produce this substance and grow as weakly negatively charged cells. They again produce this substance if grown in the presence of the drug.

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The deposition of DPPD in fowls' tissues as assessed by a new quantitative method

I. Ascarelli, P. Budowski and A. Bondi, *Faculty of Agriculture, The Hebrew University of Jerusalem*

The anti-oxidant N-N^1 diphenyl-*p*-phenylene diamine (DPPD) was at one time widely used for the stabilization of carotene in dehydrated meals and generally for the protection of other easily oxidizable nutrients as fat, Vitamin A, etc. in mashes.

According to some reports, DPPD causes reproductive failure in rats and accordingly the Food and Drug Administration of the U.S. Department of Health recommended the discontinuation of its addition to poultry mashes. In order to contribute to a better assessment of the danger to man involved in the use of this antioxidant as a feed additive to poultry mashes, a sensitive quantitative method has been developed for its determination, and its content has been determined in eggs and in the organs of chickens and laying hens fed different levels of this antioxidant.

The analytical method, applicable to a variety of biological materials as feeds, fat, organs and egg yolk, consists of a simple phasic separation, taking advantage of the fact that DPPD is a weak base soluble in strong mineral acid with salt formation. Such a salt is readily hydrolysed by the simple process of diluting the acid with water, and the base is set free again.

DPPD is accumulated mainly in the liver and in body fat. The maximum level in eggs and organs seems to be reached after two weeks' feeding, while all stored DPPD practically disappears two weeks after the feeding of DPPD has been stopped.

The absolute amount of DPPD deposited in eggs and in the carcass of birds fed the usual amount (0.0125%) of the antioxidant seems to be very small.

Levels of lysine and methionine in portal blood of rats following protein feeding

S. Halevy, Naomi Friedmann and K. Guggenheim, *Laboratory of Nutrition, The Hebrew University–Hadassah Medical School, Jerusalem*

In an attempt to correlate the biological availability of the most limiting amino acids of certain food proteins with their concentrations in the respective proteins, the levels of lysine and methionine were examined in the blood of the vena portae of rats following protein feeding. The effect of wheat gluten, zein, soya protein, casein, and lactalbumin was studied. Extent and duration of the rise of lysine and methionine in portal plasma was not in complete agreement with the content of these amino acids in the protein sources.

Experiments with unheated, heat-processed and overheated soya meals showed that lysine and methionine levels in portal blood depend not only on their amounts in the food proteins but also on their digestive release and absorption.

Feeding different carbohydrates along with soya proteins leads to different responses in lysine and methionine levels in portal plasma. Lactose, fructose and sucrose induce a greater increase of lysine level than starch and glucose, and starch, lactose and fructose cause a higher rise of methionine than sucrose and glucose.

Seventh Session Chairman: F. Bergmann

A synthetic polypeptide with the properties of a specific antigen

M. Sela and R. Arnon, *Department of Biophysics, The Weizmann Institute of Science, Rehovot*

An investigation of the antigenicity of various polypeptidyl gelatins[1] showed that the enrichment with tyrosine, tryptophan and phenylalanine converted gelatin into relatively powerful antigens, as followed by the precipitin reaction. The attachment of cysteine to gelatin caused a limited enhancement of antigenicity. Antibodies to polyalanyl, polyseryl, polyglutamyl and polylysyl gelatins could not be detected by the usual precipitin techniques, but their formation could be shown by a method similar to that used by Maurer[2] to demonstrate the antigenicity of gelatin.

It was concluded from a study of the antigenic specificity of gelatin derivatives enriched with varying amounts of tyrosine and glutamic acid[3] that the attachment of a very limited amount of tyrosine residues enhances dramatically the antigenicity of gelatin, without essentially changing its antigenic specificity. On the other hand, an increase in the amount of tyrosine, or tyrosine and glutamic acid, bound to gelatin results in changes not only in the extent of antigenicity but also in the serological specificity of the gelatin derivatives obtained. The question arises in these cases whether gelatin still contributes in any way towards the immunological properties of the new antigens.

We have now been able to demontrate that a multichain copolymer, in which chains of polypeptides containing L-tyrosine and L-glutamic acid were built on a multichain poly-DL-alanine (average molecular weight 180,000)[4], is a powerful and sharply specific antigen in rabbits. The antibodies formed could be demonstrated by the usual precipitin techniques. Approximately 500 μg of the antibody may be precipitated from 1 ml. antiserum in the equivalence zone. Except for the homologous antigen, the antibodies could be cross-precipitated only with the related copoly (tyrosyl, glutamyl) gelatin. The homologous reaction could be inhibited completely only by a copolymer of L-tyrosine and L-glutamic acid in a residue molar ratio of 1:1.

Multichain poly-DL-alanine, as well as a linear copolymer of L-tyrosine and L-glutamic acid in a residue molar ratio of 1:1 (degree of polymerization 31), failed to produce antibodies detectable by the homologous precipitin test or by cross-precipitation with related polypeptidyl derivatives.

All the specific antigens described till now are either natural substances (proteins, polysaccharides) or were derived from them by chemical modification. The above described material seems to be the first instance of a completely synthetic, highly specific antigen.

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Isolation of antibodies from antigen- antibody complex by selective proteolysis of the antigen

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The enzymatic cleavage of the antigen in the antigen-antibody complex would be a very convenient method for the isolation of antibodies, if such treatment would not affect the antibody molecules. We have now succeeded in isolating rabbit antibodies to gelatin by digestion of the gelatin-antigelatin complex with collagenase[1], and removal of the gelatin fragments by dialysis (as followed by hydroxyproline analyses[2] of the hydrolysates). The antigelatin was pure and undamaged, as followed by electrophoresis and by sedimentation in the ultracentrifuge. The pure antibody could be precipitated anew by the antigen.

Antibodies to tyrosylgelatin (a material containing 2% tyrosine residues), a strong antigen in comparison to gelatin[3], were isolated in a similar way, but in this case peptides containing the active antigenic sites remained bound to the antibody and thus prevented reprecipitation with new antigen.

The above conclusions are supported by the results of inhibition experiments of the specific precipitin reaction by means of collagenase digests of the antigens. While the collagenase digest of gelatin does not inhibit the precipitation of antigelatin by gelatin, it was found that the collagenase digest of tyrosylgelatin completely inhibited the precipitation of antityrosylgelatin by the homologous antigen.

The method used here for the antibody purification is limited to systems where an enzyme may digest the antigen in the antigen-antibody complex without damaging the antibody molecules. The active site of the antigen may under these circumstances remain, in some cases, bound to the antibody. This might permit the isolation of the active fragment.

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Isolation of an antigenic surface compound from *Pasteurella pestis* with enzymatic activity

LEAH BICHOWSKY-SLOMNICKI AND S. BEN-EFRAIM, *Department of Biophysics, The Weizmann Institute of Science, Rehovot and Israel Institute for Biological Research, Ness Ziona*

It was previously reported[1,2] that virulent as well as nonvirulent *P. pestis* cells incubated at *p*H's below 6.7 and temperature above 35°C undergo a decrease in their surface charge as shown by changes in their electrophoretic mobility. This change was found to be connected[3] with the synthesis of a new surface antigen, which could be detected by the gel diffusion technique. The synthesis of this component also occurred *in vivo* in experimental animals inoculated with *P pestis* cells previously devoid of this antigen. In the present paper we wish to report further steps in the isolation, purification and biological characterisation of this antigen.

P. pestis TRU avirulent cells were incubated in casamino-salt-xylose medium at *p*H 6 at 37°C for 3 hours. The cells were centrifuged off and the extraction of the antigen was performed with 1M KSCN at *p*H 7.5 under constant shaking at room temperature for 5 hours. The extract was dialysed in the cold against distilled water. When the salt concentration inside the dialysis bag reached 0.04M, a precipitate formed and was collected by centrifugation. By precipitation with ammonium sulphate, 0 to 50% saturation and purification by resin electrophoresis a protein fraction was obtained. In gel diffusion this fraction gave only one precipitation line against anti-serum prepared by injection of whole cells. The same fraction was biologically active *in vivo* and *in vitro*. A marked inflammatory reaction with oedema, swelling and necrosis appeared in rabbits after intradermal injections of this component. Liquefaction and hydrolysis of gelatin was noted *in vitro*. Both of these reactions were inhibited by specific anti-serum. The activity disappeared after heating for 15 minutes at 56°C.

The physico-chemical and biological properties of this antigen are under study. The possible significance of these data in the pathogenesis of plague will be discussed.

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Poisoning and sign reversal of perm-selective membranes; a possible model for physiological membrane phenomena

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Electromotive forces across biological membranes are the basis of electric impulses along nerve fibres and are the cause, as well as the effect, of chemical reactions at the end of the fiber. The signs transmitted by the nervous system are rapid changes in the potential. Primarily, these electrical impulses are generated by changes in the chemical environment of sensitive portions of the nerve. Any analogy comprising changes of EMF across man-made membranes induced by changes in the chemical composition of solutions on its sides, might be helpful in getting nearer to the working mechanism of physiological membranes.

EMF changes caused by simple change of conncetration of ions are relatively well understood. There exists, however, another mechanism based on changes in the internal composition of the membrane. Working on the synthetic perm-selective membranes for the purpose of electrodialytic water desalination, we have been confronted with the known effect of membrane poisoning by highly charged counter-ions. These decreased the EMF across the membrane, which was between two ionic solutions of different concentrations. In special cases we were even able to reverse the sign of the E.M.F. There seems to be an analogy between these *in vitro* phenomena and the behaviour of biological membranes, which would certainly merit further investigation. For example, curare-like poisons with two positively charged groups in their molecule, block the nerve-end plates which originally operate with single-charged choline.

It has already been found by J. H. George[1] that electro-osmotic water transfer across cation active membranes becomes negative (from cathode towards anode) between $ThCl_4$ solutions of different concentrations. This was explained by the formation of Th complexes within the membrane, effectively changing it to an anion exchanger. Very low Th transference was found.

We ascertained the sign reversal of the anion-selective membrane by working with identical Th^{++++} (or other poisons) concentration, but different KCl concentrations on its two sides. Thus all factors resulting from the different mobility of Th^{++++} and Cl^- ions are eliminated; Th only affects the membrane and KCl shows that its sign has been reversed.

The same effect was observed with cation-selective membranes in the presence of $K_4Fe(CN)_6$ and $K_3Fe(CN)_6$. Other ions of lower charge or of charge less concentrated on a single location within the molecule, were active in as far as they much decreased the E.M.F. of the membrane, but they failed to reach the point of sign reversal.

On increasing the concentration of the "poisoning" ions the original E.M.F. of the membrane between two KCl solutions of different concentrations begins to decrease, falls to zero and then changes its sign. The necessary concentration of poison is smaller in diluted KCl than in concentrated KCl solutions, because of their competitive effects for the active sites of the membrane.

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Activation of glucose-6-phosphate dehydrogenase of erythrosytes by a stromatic factor

Abraham Rimon, Israel Ashkenazy and Bracha Ramot, *The Marcus Memorial Blood Institute-Magen David Adom in Israel, Jaffa and Tel-Hashomer Government Hospital*

A decreased activity of glucose-6-phosphate dehydrogenase (G-6-P.D.) was observed in the erythrocytes of Negroes, Oriental Jews and Italians, susceptible to hemolysis, following the administration of primaquine, sulfanilamides and other drugs as well as fava beans, Those subjects will be referred to as "Sensitive". After haemolysis, G-6-P.D. is found in the stroma free haemolysate.

In the present investigation the effect of erythrocyte stroma on the enzyme activity was studied. This was carried out by pre-incubating a red cell heamolysate with stroma at 37° for 1 hour. The enzymatic activity of G-6-P.D. was determined before and after the preincubation, by measuring the reduction of TPN. It was found that erythrocyte stroma of normal subjects is able to increase the enzymatic activity of "sensitive" haemolysates. Such an activation was not observed with stroma of "sensitive" subjects. (Table 1).

TABLE I

*The effect of stroma on G-6-P.D. activity in haemolysates.**

Reaction mixtures		Activity ($\triangle$OD/min/gr H_b)	
Stroma	Haemolysate	No preincubation	1 hour preincubation
——	normal	18	18
——	"sensitive"	1.5	1.5
normal	normal	18	18
"sensitive"	"sensitive"	1.5	1.5
"sensitive"	normal	18	18
normal	"sensitive"	1.5	17

* A typical experiment.

The activation of the enzyme by the stromatic factor is temperature and *p*H dependent and progresses with time.

The kinetic behaviour of the activation effect and its temperature and *p*H dependence support the assumption that it is of an enzymatic nature.

Eighth Session Chairman: D. Elson

Electromicroscopic studies of cellulose fibre formation by washed cells of *Acetobacter xylinum*

Itzhak Ohad, *Department of Biological Chemistry, The Hebrew University of Jerusalem* and David Danon, *Polymer Department, Laboratory of Electronmicroscopy, The Weizmann Institute of Science Rehovot*

Electronmicrographs of fibre formation by washed cell suspensions of *Acetobacter xylinum* will be presented and discussed.

Hydrodynamic properties of poly-ε-carbobenzoxy-L-lysine in solution

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Seven samples of polycarbobenzoxy-L-lysine[1] have been prepared and their sedimentation, diffusion and viscosity in dimethylformamide determined. The samples tested had average molecular weights in the range of 20,000 to 600,000 (see Table). A single symmetrical peak was observed in the ultracentrifuge in all cases. The intrinsic sedimentation and diffusion constants as well as the intrinsic viscosities given were derived from the corresponding measurements at different concentrations after making the suitable extrapolation.

TABLE I

Sample number	s_0 in Svedbergs	$[\eta]$ in 100 g/g	$D \times 10^7$ in cm²sec⁻¹	Average molecular weight	Axial ratio
XV	1.90	0.06	11.0	21,000	7
XIII	2.15	0.17	6.5	41,000	13
XIV	2.65	0.21	5.2	64,000	16
III	2.90	0.37	4.1	88,000	22
XI	3.20	0.74	2.0	200,000	34
XII	3.75	1.38	1.8	260,000	48
VII	3.70	2.50	0.8	578,000	68

The following viscosity-molecular weight relatioship was found to hold:

$$[\eta] = 6.0 \times 10^{-6} M^{1.2}$$

The axial ratios given in the last column of the table represent the axial ratios of the prolate equivalent ellipsoids and were calculated from the viscosity data by means of the Simha[2] equation. These compare well with the axial ratios calculated by the Perrin[3] equation from D and M.W. The axial ratios obtained for the various polycarbobenzoxy-L-lysines indicate that this polypeptide behaves in dimethylformamide solutions as an α-helix with a limited degree of flexibility.

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Synthesis and properties of some multichain polyamino acids

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In a previous report[1] a description was given of the preparation of multipoly-β-benzyl-aspartyl-polylysine and of its use as a dioxane-soluble polyfunctional initiator in the preparation of multichain polyamino acids. The hydrodynamic properties in dimethylformamide of two multichain initiators as well as of the corresponding two branched γ-benzyl-glutamates derived from them is given in the Table. The high sedimentation constants and the relatively low intrinic viscosities of all the polymers tested indicate that multichain polyamino acids possess in solution hydrodynamic properties similar to those of globular molecules.

TABLE

*Hydrodynamic properties of multipoly-benzyl-*L*-glutamyl-benzyl-*L*-aspartyl-poly-*L*-lysine in dimethylformamide.*

D.P. of backbone	Number of amino acid residues per side chain		M.W.	$[\eta]$ $\frac{100\text{ ml}}{\text{g}}$	*So* Svedberg units
	Aspartic acid	Glutamic acid			
70	3.5	—	59000	0.06	3.8
70	3.5	12	243000	0.30	5.7
140	3.3	—	112000	0.16	6.0
140	3.3	10	419000	0.30	11.0

A histidine-containing copolypeptide was prepared by coupling carbobenzoxyhistidine azide with multi-polybenzyl-aspartyl-polylysine. Removal of the protecting groups leads to an ampholytic multichain copolymer possessing on the average three aspartyl residues and one histidine residue per side chain. The multichain ampholyte migrated towards the anode at *p*H 6.0 and gave a positive reaction with ninhydrin and with the Pauli reagent. Potentiometric titrations showed the presence of three buffering regions characteristic for the β-carboxyl, the imidazole and the α-amino groups.

Branched copolymers with an optically inactive backbone and poly-L-proline side chains of different chain length were also synthesized. Even a copolymer containing six L-proline residues per side chain showed the typical mutarotation of poly-L-proline[2,3].

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Catalysis of the mutarotation of poly-L-proline by strong acids

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Two forms of poly-L-proline have been described[1]. Form I of the polymer exhibits a specific optical rotation $[\alpha]_D = +50°$ in acetic acid, whereas form II shows $[\alpha]_D = -540°$ in this solvent. On standing in formic aid or acetic acid, form I mutarotates into form II[1]. Dissolution of form II in acetic acid-propanol (1:9 v/v) leads to a reverse mutarotation resulting in the formation of form I[2].

Strong mineral acids, such as perchloric acid, were found to enhance the forward, as well as the reverse, mutarotation. For example, 0.052 mole perchloric acid per mole peptide bond reduced the half life time of the forward mutarotation (I → II) in glacial acetic acid, at 30°, from 345 to 4 minutes; the half life time of the reverse mutarotation (II → I) in water-propanol (1:9 v/v), at 40°, was similarly reduced, in the presence of 0.15 M perchloric acid, from 445 to 60 minutes.

The attachment of the perchloric acid to the imide bonds of poly-L-proline could be demonstrated by the formation of polyproline-perchloric acid precipitates in acetic acid, as well as by the potentiometric titration of the prolyl-imide bonds in acetic anhydride.

The suggestion was made that form I consists of a right-handed helix whose peptide bonds are arranged in a cis-configuration[3], while form II consists of a left-handed helix whose peptide bonds are arranged in a trans-configuration[4]. The acid catalysis discussed provides important evidence for the occurrence of cis-trans isomerizations during the mutarotation. Protonation of the prolyl-peptide nitrogen eliminates the double bond character of the $-\mathrm{CON}<$ bond, thus facilitating the isomerization reaction.

The kinetics of forward mutarotation of Form I in acetic acid and reverse mutarotation of form II in acetic acid-propanol have been examined at several temperatures between 30° and 45°. From this information an enthalpy of activation of about $\triangle H^* = 20$ kcal. per mole prolyl residue was calculated for both reactions. The value obtained is consistent with the assumption that the elimination of the double bond character of prolyl-peptide bonds[5] occurs as an intermediate step in the mutarotation process.

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Hydrogen bonds in polyserine

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The most striking difference between poly-L-serine and poly-DL-serine lies in their solubility in water. Poly-L-serine may be obtained in a form which is not soluble at all, whereas poly-DL-serine is freely soluble. These polyamino acids were prepared from the *o*-benzylserine carbamino anhydrides by polymerization in boiling chloroform, using triethylamine as initiator. The benzyl groups were removed by suspending the polymers in dioxane saturated with hydrogen bromide and allowing to stand in the cold.

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* On sabbatical leave from the University of Pittsburgh, Pittsburgh, Penna., U.S.A. U.S. Public Health Service Special Fellow 1959—1960.

Two preparations of polymers were studied. End group analysis of the poly-*o*-benzyl-L-serine indicated a degree of polymerization of 90. For poly-DL-serine the degree of polymerization was estimated as 100 on the basis of ultracentrifuge studies. In solutions of 0.15 molar sodium chloride S_{20_w} was independent of the concentration and had a value of 1.50 S. Infrared spectra were determined in potassium bromide pellets. A comparison of the observed frequencies for the most important modes is shown in Table I.

TABLE I

Infrared absorption bands for polyserine (in cm^{-1})

Poly-L-serine	poly-DL-serine ($\frac{1}{2}H_2O$ per residue)	liquid Water	Assignment
3,370	3,490	3,450	OH — s
3,240	3,360	3,330	OH — s
3,140			OH — s
2,920	3,000		CH — s
1,650	1,767	1,653	OH — d
1,621	1,695	1,613	OH — d
1,675	1,675		CONH
1,399	1,422		CH_2 — d
730	816		CH_2 — r

These assignments are based on previous studies of infrared spectra of amino acids and peptides as solids[1], as well as on studies of the infrared spectra of their aqueous solutions[2]. Whereas solid samples of such compounds have absorption bands in the 3,000 to 3,600 cm^{-1} and 1,580 to 1,680 cm^{-1} regions due to modes associated with the NH- group, samples of aqueous solutions have such bands only when the compounds exist as $NH_2CHRCOO^-$. As soon as amino acids or peptides acquire the NH_3^+-group, the phenomenon of "negative absorption" is observed. In this case, the solutions are more transparent in the above ranges than the reference cell containing water. It was shown that the "negative absorption" is due to the formation of a hydrogen-bonded complex between solute and water[2].

It is seen from Table I that the bands due to water of hydration and free OH-groups in poly-DL-serine occur where expected, whereas in poly-L-serine they are shifted to lower frequencies. In addition, the CH- and CH_2-modes in poly-L-serine are shifted to lower frequencies than in poly-DL-serine, in good agreement with a tightly hydreogn bonded configuration. It is concluded that poly-L-serine has many more hydrogen bonds than poly-DL-serine, and that the presence of a tightly hydrogen-bonded configuration is responsible for the insolubility of poly-L-serine in water.

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Preparation and UV spectra of benzoyl-(polyglycine)-anilides and benzoyl-(polyalanine)-anilides

ELIAHU HOFFMANN* AND A. R. GOLDFARB, *Department of Organic Chemistry, The Hebrew University of Jerusalem and University of Chicago Medical School*

The literature contains certain indications as to the possibility of energy transfer through a peptide chain. The present paper attempts to detect this effect by spectroscopical methods.

If one prepares a series of simple polypeptides with identical chromophores attached to both ends of the polypeptide chain, an interaction between these groups through the peptide chain, if operative, would be detectable in the UV spectra of these compounds, and upon lengthening the chain by additional peptide groups, a gradual change in the UV spectrum should be noticeable. Such phenomena are well known, e.g., in the α,ω-diphenylpolyene series, where a gradual lengthening of the *conjugated* chain produces a marked red-shift in the UV absorption spectra.

We prepared by unambiguous, but conventional methods a series of benzoyl-(polyglycine)-anilides, starting with benzoyl-glycine-anilide and up to and including benzoyl-(hexaglycine)-anilide, and further benzoyl-alanine-anilide and benzoyl-(dialanine)-anilide. The UV spectra of these compounds showed a distinct hypsochromic shift with increasing length of the peptide chain. Benzoyl-glycine-anilide absorbs at 240 $m\mu$; this peak is shifted towards the blue until a value of 225 $m\mu$ is obtained for the absorption maximum of the benzoyl-(hexaglycine)-anilide.

A tentative explanation for these experimental results will be proposed.

Ninth Session Chairman: E. KATCHALSKI

The influence of ethyl carbamate (urethan) on nucleic acid metabolism

ALVIN M. KAYE, *Department of Experimental Biology, The Weizmann Institute of Science, Rehovot*

A study has been initiated to discover whether specific changes in the pattern of incorporation of radioactive nucleic acid precursors can be caused by the presence of urethan in the animal body, and whether urethan inhibits, *in vitro* any of the enzymatic steps in nucleic acid biosynthesis.

An incorporation of thymine-2-C^{14} into mouse DNA thymine was found and the influence of urethan on this incorporation and on the incorporation of orotic acid-6-C^{14} into mouse nucleic acid bases will be reported.

No interaction between urethan and carbamyl phosphate was found in tests on carbamyl phosphate synthetase, ornithine transcarbamylase, asparatate transcarbamylase or the enzyme system which catabolizes urethan[1]. Tests on other steps in nucleic acid synthesis will be reported.

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* Lecturer

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Ribonucleotide incorporation into ribonucleic acid with an enzyme from rat liver

V. DANIEL AND U. Z. LITTAUER, *The Weizmann Institute of Science, Rehovot*

An enzyme which incorporates 8-C^{14} labeled adenosine triphosphate into soluble ribonucleic acid (RNA) of rat liver was isolated and purified. The rat liver homogenate was fractionated by differential centrifugation and the particle-free cytoplasmic supernatant was obtained. This fraction contained the ribonucleotide-incorporating system and the soluble RNA. The enzyme was purified about 150 fold by precipitation at *p*H 5, removal of the RNA with streptomycine, ammonium sulfate fractionation, and calcium phosphate gel adsorption.

On removing the RNA, the enzyme activity was lost. The activity was restored by addition of the streptomycine precipitate or the soluble RNA prepared by the phenol method[1,2] from the cytoplasmic supernatant of rat liver homogenate.

This RNA was highly specific; it activated the enzyme about 40 fold and could not be replaced by rat liver microsomal RNA or by bacterial RNA (*E. coli*, *B. cereus*) It revealed a single somewhat broad peak in the ultracentrifuge, $S_{20} = 3.9$ in 0.2 M NaCl containing 0.05 M phosphate buffer, *p*H 6.8.

The purified enzyme required Mg^{++} in the proportion of 2 μmoles $MgCl_2$/mg RNA, and its optimal activity was at *p*H 9.5. ADP did not replace the ATP in the reaction. One mg of the purified enzyme preparation incorporated 3.0 μmoles ATP per hour at 37°. The reaction could be formulated according to the equation

$$\mathrm{ATP}+\mathrm{RNA}\rightleftharpoons\mathrm{RNA{-}AMP}+\mathrm{PP}$$

The reversibility of the reaction was shown by P^{32} P^{32} exchange into nucleoside triphosphates incubated in the presence of the enzyme and RNA. Inorganic orthophosphate was inactive. The location of the incorporated ATP in the RNA chain was also determined.

(This research was supported in part by a U.S. Public Health Service research grant RG-5217).

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Biochemical differences in ribonucleoproteins

M. TAL AND D. ELSON, *Biochemistry Section, The Weizmann Institute of Science*' *Rehovot*

Ribonucleoprotein (RNP) particles were isolated from *Escherichia coli* by differential ultracentrifugation. In a medium containing 0.002 M Mg acetate, the principal component is a particle of sedimentation constant 70 S. In 0.001 M Mg acetate and 0.01 M neutral phosphate buffer, this particle dissociates reversibly into two particles of sedimentation constant approximately 50 S and 30 S[1]. Attempts to separate the 50 S and 30 S particles from each other by differential ultracentrifugation were not completely successful. Nearly pure preparations were obtained, but each particle was slightly contaminated with the other. All three particles contained about 60% RNA and 40% protein by weight.

RNP prepared by a different technique is known to contain two latent enzymes, ribonuclease (RNase) and deoxyribonuclease (DNase)[2]. The RNP particles prepared by ultracentrifugation also contain these enzymes. Quantitative assays showed that the 70 S and 30 S particles possessed high RNase activity. The RNase activity of the 50 S particles was low and could be accounted for by the small fraction of contaminating 30 S particles.

In order to clarify this point, we prepared mixtures containing 50 S and 30 S particles in different proportions. These mixtures were obtained from the precipitates resulting from successive ultracentrifugations of a mixture of the two particles. Each precipitate was thus richer in 30 S particles than the earlier precipitates. The relative amounts of 30 S and 50 S particles in each mixture were determined in the analytical ultracentrifuge.

A plot of the specific RNase activity of the different mixtures as a function of composition of the mixtures gave a straight line extrapolating to zero activity for pure 50 S particles. Thus, it appears that all or virtually all of the RNase is located in the 30 S particle. Preliminary experiments indicate a similar distribution for the DNase.

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Antibodies in ribonucleoproteins

D. ELSON, M. FELDMAN AND A. GLOBERSON, *The Weizmann Institute of Science, Rehovot*

Although it has been suggested that the nucleic acids are involved in the production of antibodies, no clear evidence for their participation is available. There is reason to believe that the ribonucleoproteins (RNP) play a role in general protein synthesis. The present communication describes experiments which indicate that the RNP of the antibody forming organs (spleen and lymph node) is involved in the production of specific antibodies.

Rabbits were immunized against sheep erythrocytes. When the animals showed a high serum antibody titer, the spleens were removed and RNP was isolated by standard procedures. The RNP was then digested with ribonuclease and centrifuged.

When the clear supernatant fluid was tested with the antigen, a typical agglutination reaction occurred. A typical positive Coombs test was also observed. If the RNP had not been digested with ribonuclease, there was no reaction. Controls with non-immunized animals also gave negative results. Similar results were obtained with lymph node RNP isolated from rabbits immunized against horse serum globulin, except that the presence of these antibodies could be demonstrated only by indirect tests (passive agglutination of tanned erythrocytes coated with antigen).

The results indicate that the RNP contains antibodies which are released in active form upon the destruction of the nucleic acid moiety. This is consistent with the concept that the antibody molecule forms part of the RNP complex and may be synthesized there. Since similar observations have been made in the case of the release of certain enzymes from a bacterial RNP, it is possible that this is a general phenomenon of protein biosynthesis.

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GLUTAMINE SYNTHETASE AND GLUTAMOTRANSFERASE IN SHEEP LIVER ENZYME PREPARATIONS*

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ABSTRACT

Sheep liver preparations which contain a glutamotransferase system also contain a glutamine synthesizing system. Both systems can be activated with Mn^{++}. As in the case of the corresponding sheep brain enzymes, the *p*H optimum of both systems is higher at low Mn^{++} concentrations and lower at high Mn^{++} concentrations. In the case of glutamine synthetase it varies from *p*H 4.5 to *p*H 6.5; in the case of transferase it varies from *p*H 5.5 to *p*H 7.2.

Similarly to sheep brain glutamine synthetase, sheep liver glutamine synthetase can be activated with Mg^{++}. The *p*H optimum varies in this case from 6.6 at higher Mg^{++} concentrations to *p*H 8 at lower Mg^{++} concentrations. As in the case of the brain enzyme, optimal activity of Mg^{++}-activated sheep liver glutamotransferase occurred at about 7.2.

In a previous study[1] it was shown that the *p*H optimum of the Mn^{++}-activated sheep brain glutamine synthetase depended on the Mn^{++}- concentration, varying from 4.5 at high Mn^{++} concentrations to 6.5 at low Mn^{++} concentrations. Mn^{++} concentrations which were strongly inhibitory in the neutral region readily activated the synthesis at *p*H values of 5 and 4.5. When the glutamine synthesizing system was activated by Mg^{++}, optimal activity occurred between *p*H 6 to 7.2 at higher Mg^{++} concentrations whereas at low Mg^{++} concentrations the optimum was shifted to *p*H 8. Mg^{++} concentrations optimal for *p*H 7.2 did not activate the synthesis at *p*H 5 and at *p*H 4.5. The *p*H optimum of the Mn^{++}-activated sheep brain glutamotransferase also depended on the Mn^{++} concentration. It was higher at low Mn^{++} concentrations and lower at high Mn^{++} concentrations and varied from *p*H 5.5 to *p*H 7.2. At *p*H 7.2 the transferase system could also be activated with Mg^{++}.

Schou *et al*[2] found that acetone-dried sheep liver preparations contained a Mn^{++}-dependent glutamotransferase system. In view of the results of our experiments with the sheep brain enzymes[1], it appeared of interest to study whether the sheep liver preparations also contain a glutamine-synthesizing system, and if they do, to investigate its properties with respect to activation with Mn^{++} and Mg^{++}. The present paper deals with this problem. The effect of Mn^{++} and Mg^{++} concentrations on sheep liver glutamotransferase is also described.

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EXPERIMENTAL

The enzyme preparations used in the present investigation were obtained from acetone-dried sheep liver in the same way as the sheep brain preparations used in the previous study[1]. They were free from ATP-ase activity at all *p*H values and Mn^{++} and Mg^{++} concentrations used. One ml of enzyme solution corresponded to 0.4 to 0.8 g of acetone-dried sheep liver. The materials used (glutamic acid, glutamine, ATP and ADP) and the analytical methods employed were the same as described in the previous communication[1].

RESULTS

As can be seen from Figure 1, increased Mn^{++} concentration shifts the *p*H dependence curve of the Mn^{++}-activated glutamine synthetase to the left. This shift is accompanied by an elevation of the peak up to *p*H 4.5. At this *p*H the hydroxamic acid value obtained is higher than the value obtained under optimal conditions with Mg^{++} as activator. The *p*H optimum of the Mg^{++}-activated synthesizing reaction varies from 6.6 at higher Mg^{++} concentrations to 8 at lower Mg^{++} concentrations (Figure 2). One can see from the table that the hydroxamic acid values observed at

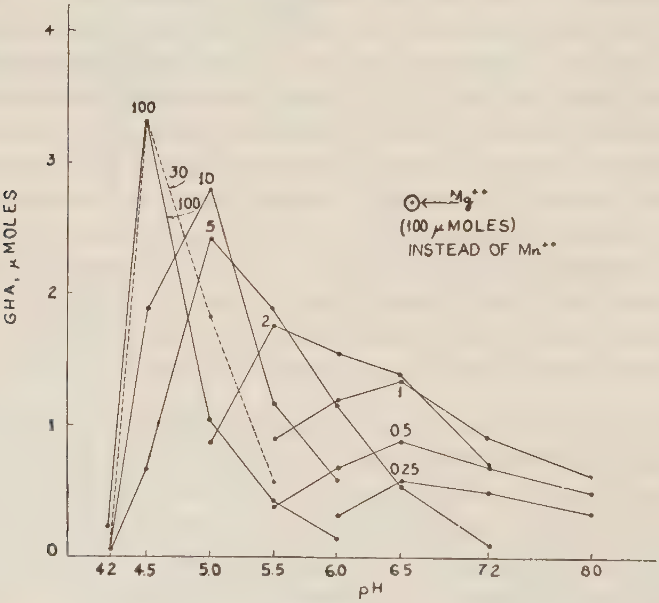

Figure 1

Effect of Mn^{++} concentration on glutamine synthetase activity. Each reaction mixture contains, in 4.5 ml of buffer (0.08 M acetate buffer for *p*H values below 7; 0.04 M tris (hydroxymethyl) aminomethane buffer for *p*H values above 7): L-glutamic acid, 100 μmoles; $NH_2OH \cdot HCl$, 50 μmoles; ATP, 5 μmoles; enzyme solution, 1 ml; $MnCl_2$, μmoles, as indicated above for each curve. All solutions added were adjusted to the appropriate *p*H with NaOH. Incubation was at 30° for 20 minutes. ATP was added after 5 minutes preincubation (same conditions in Figure 2 and Table). GHA, glutamohydroxamic acid.

TABLE

Glutamohydroxamic acid formed and inorganic phosphate liberated in the Mn^{++} or Mg^{++} – activated sythesizing reaction

Reaction mixtures are as given in Figure 1. The phosphate values are corrected by subtraction of blanks.

$MnCl_2$ μmoles	$MgSO_4$ μmoles	*p*H	Glutamohydroxamic acid formed μmoles	Phosphate liberated μmoles
1		7.2	0.5	0.4
5		7.2	0.1	0.05
10		7.2	0.1	0.05
100		7.2	0.1	0
1		5.0	0.1	0.05
5		5.0	1.25	1.3
10		5.0	1.55	1.55
100		5.0	0.6	0.65
1		4.5	0.1	0.1
5		4.5	0.4	0.35
10		4.5	0.7	0.75
100		4.5	1.8	1.9
1; 5; 10; 100		4.2	0	0
	5	9.0	0.3	0.4
	10	9.0	0.4	0.45
	200	9.0	0.45	0.4
	5	8.0	0.9	0.9
	10	8.0	1.25	1.2
	200	8.0	1.3	1.4
	5	6.6	0.4	0.5
	10	6.6	1.1	1.0
	200	6.6	2.3	2.3
	5	5.5	0	0
	10	5.5	0	0
	200	5.5	0.7	0.6

varying *p*H values correspond to the amounts of inorganic phosphate liberated in the synthesizing reaction.

In the Mn^{++}-activated transferase reaction increased Mn^{++} concentration also ishfts the whole *p*H dependence curve to the left. The *p*H optimum varies from 7.2 to 5.5 (Figure 3). The transferase could also be activated, although rather weakly, with Mg^{++}. In this case the *p*H optimum lies at about 7.2 (Figure 4).

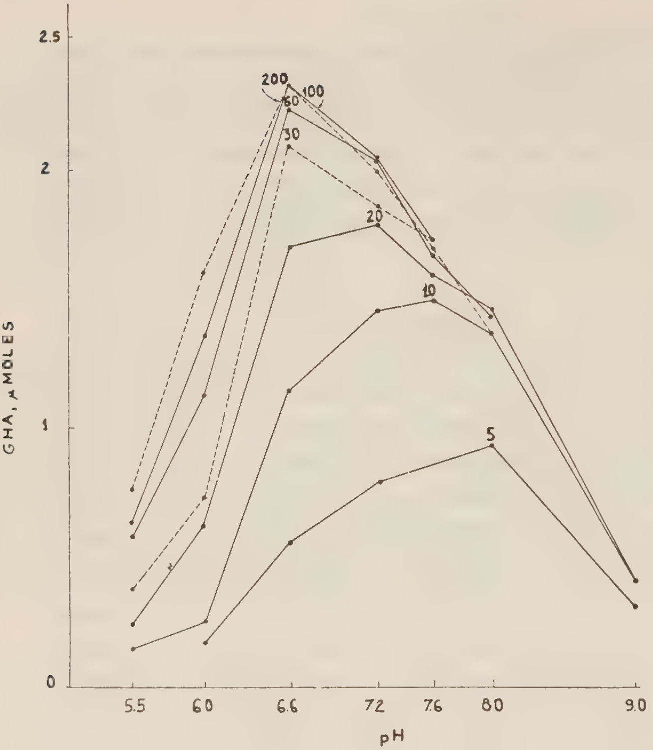

Figure 2

Effect of Mg^{++} concentration on glutamine synthetase activity. L-glutamic acid, NH$_2$OH, ATP/enzyme solution, and buffer, as given in Figure 1. MgSO$_4$, μ moles, as indicated above for each curve. GHA, glutamohydroxamic acid.

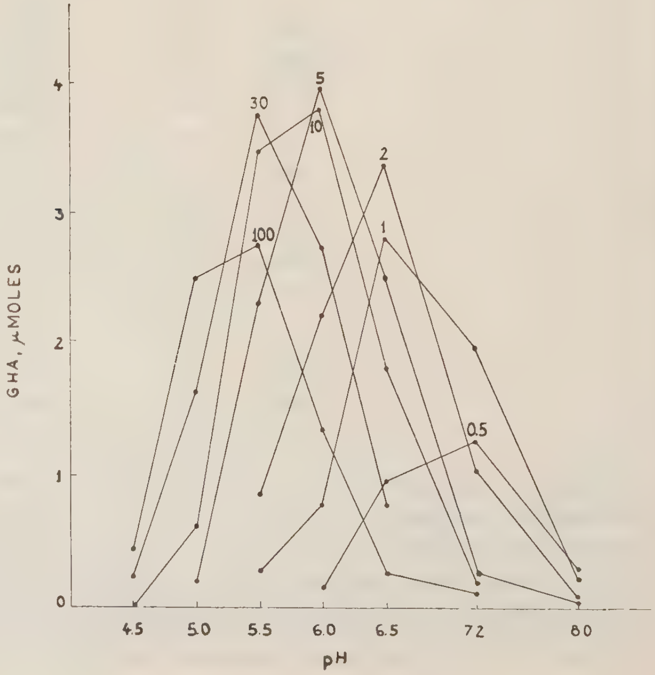

Figure 3

Effect of Mn^{++} concentration on glutamotransferase activity. Each reaction mixture contains, in 4.5 ml of buffer (as in Figure 1): L-glutamine, 100 μmoles; NH$_2$OH · HCl, 50 μmoles; ADP, 2.5 μmoles; enzyme solution, 1 ml; MnCl$_2$, μmoles, as indicated above for each curve. Incubation, as in Figure 1. Glutamine was added after 5 minutes preincubation (same conditions in Figure 4). GHA, glutamohydroxamic acid.

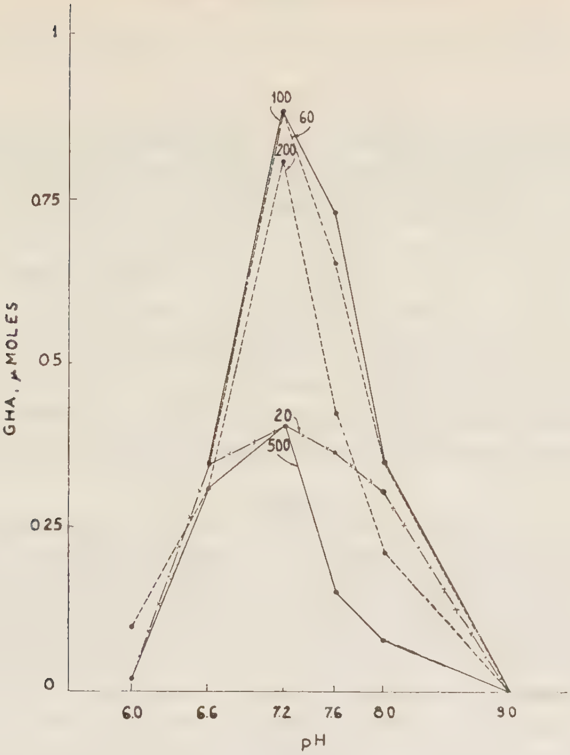

Figure 4
Effect of Mg^{++} concentration on glutamotransferase activity. L-glutamine, NH_2OH, ADP, enzyme solution and buffer, as given in Figure 3. $MgSO_4$, μmoles, as indicated above for each curve. GHA glutamohydroxamic acid.

DISCUSSION

Our experiments show that the glutamotransferase activity found in sheep liver preparations is accompanied by glutamine synthetase activity. A close association of both activities has been previously found in purified preparations from sheep brain[3], pigeon liver[3] and green peas[4,7]. Our experiments also show that, as in the case of the brain preparations, the action of Mn^{++} and Mg^{++} on synthetase and transferase activities of sheep liver preparations are quite similar. Our findings seem therefore to give an additional support to the conclusion[5–7] that glutamine synthesis and the transfer reaction are catalyzed by the same enzyme.

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OPTICAL ROTATORY DISPERSION AND VISCOSITY CHANGES ACCOMPANYING THE FORWARD AND REVERSE MUTAROTATION OF POLY-L-PROLINE IN SOLUTION

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ABSTRACT

Changes in the optical rotatory dispersion and in the viscosity of poly-L-proline during its forward and reverse mutarotation were investigated. The forward mutarotation was followed in acetic acid and acetic acid–water mixtures. It can be concluded from the results reported that while the viscosity is determined primarily by the end-to-end distance of the polymer, the optical rotation is a reflection of the helical structure of relatively short segments.

Poly-L-proline exists in two distinct forms[1,2]. Form I of the polymer exhibits a specific optical rotation $[\alpha]_D = +50°$ in acetic acid, whereas Form II shows $[\alpha]_D = -540°$ in this solvent. Each of the two forms has a regular and characteristic configurational pattern which is reflected in its hydrodynamic properties as measured by sedimentation and viscosity[3,4]. The two different homogeneous structures give differing configurational contributions to the specific optical rotation.

Poly-L-proline I mutarotates in aliphatic acids, water and other solvents into poly-L-proline II [1,2]. The reverse mutarotation of poly-L-proline II into polyproline I occurs on dilution of acid solutions with alcohols such as propanol or butanol[5]. It has been suggested that the polyproline I — polyproline II interconversion results from a series of cis — trans isomerisations at the peptide bonds of the polymer[1]. Cowan and McGavin[6] concluded from a study of the X-ray diffraction of poly-L-proline II that the molecules of form II possess a left-handed helical configuration determined by the trans structure of the peptide bonds (see also Sasisekharan[7]). The peptide bond is considered to be in the trans position when the α-carbons adjacent to it are trans one to another. Recent studies of the kinetics of the forward and reverse mutarotation of polyproline[4], taken in conjunction with the hydrodynamic data, have demonstrated that Form II retains also in solution the left-handed helical configuration suggested by Cowan and McGavin. Similar studies on the structure of poly-L-proline I suggested that the molecules in this form possess in solution a

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right-handed helical configuration determined by the cis structure of the peptide bonds[3,4].

Changes in the optical rotatory dispersion, and in viscosity, during the conversion of a low molecular weight poly-L-proline I in water into poly-L-proline II have been recorded previously[3]. In the present article we report the changes in the optical rotatory dispersion and in the viscosity of high molecular weight poly-L-proline during its forward and reverse mutarotation. The forward mutarotation was investigated in acetic acid and acetic acid–water mixtures.

MATERIALS AND METHODS

The synthesis of poly-L-proline has been described in earlier papers[8,9]. The various samples were stored in a desiccator over phosphorus pentoxide and all solutions of the polymers were made up by weight, assuming them to have a negligible water content.

Formic acid, glacial acetic acid and propionic acid were of analytical purity. *n*-Propanol and *n*-butanol were dried for a week over calcium oxide and distilled.

Optical rotatory dispersions were measured with a Rudolph precision ultraviolet polarimeter, model 80, equipped with a Rudolph photoelectric polarimeter attachment and an oscillating polarizer prism. Dispersion studies included the spectral range from 350 to 750 mμ, and were obtained through the use of a xenon compact arc lamp provided with the instrument. In some experiments water-jacketed polarimeter tubes were used; water from a thermostatted water-bath served to control the temperature within ± 0.1°.

Viscosity measurements were made with 10 ml volume Ostvald-Fenske viscometers. Temperature was controlled within ± 0.03°.

RESULTS AND DISCUSSION

The optical rotatory dispersion at the beginning and at the end of the mutarotation of a high molecular weight polyproline in glacial acetic acid has been described by Blout and Fasman[10]. Dispersion measurements of polyproline (B 4, molecular weight 18,000) at various times during the mutarotation from Form I into Form II, in glacial acetic acid at 35°, are presented in Figure 1. Because of the speed of the mutarotation it was impossible to measure the $[\alpha]_\lambda$ values at five different wavelengths fast enough to be able to relate them to a single $[\alpha]_D$ value, i.e. to a defined degree of reaction. The data of Figure 1 were, therefore, read off the graphs of the forward mutarotation, measured at different wavelengths, presented in Figure 2. All the curves of Figure 2 were drawn from the data recorded in a single mutarotation experiments.

Plots of $\lambda^2[\alpha]_\lambda$ versus $[\alpha]_\lambda$ derived from Figure 1, for the various times of the forward mutarotation in glacial acetic acid, are given in Figure 3. Straight lines, indicating a normal one Drude term dispersion, are obtained from within one hour after the beginning of the mutarotation onwards. The value of $[\alpha]_D$ after one hour

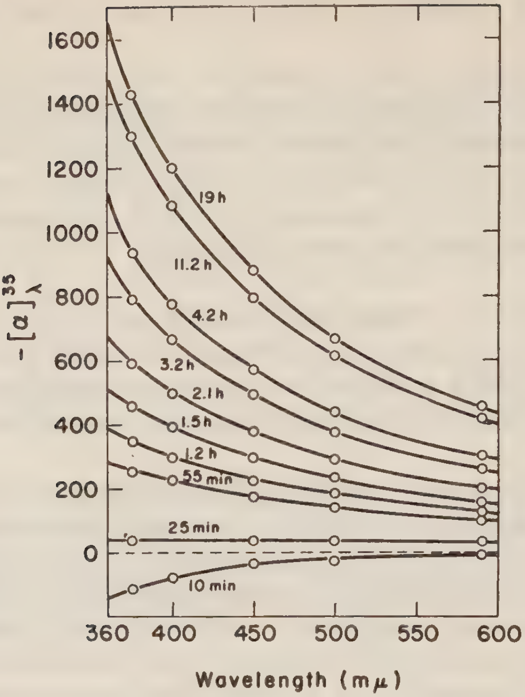

Figure 1.
Optical rotatory dispersion of poly-L-proline (B 4, molecular weight 18,000) during the mutarotation from Form I to Form II, in glacial acetic acid at 35°.

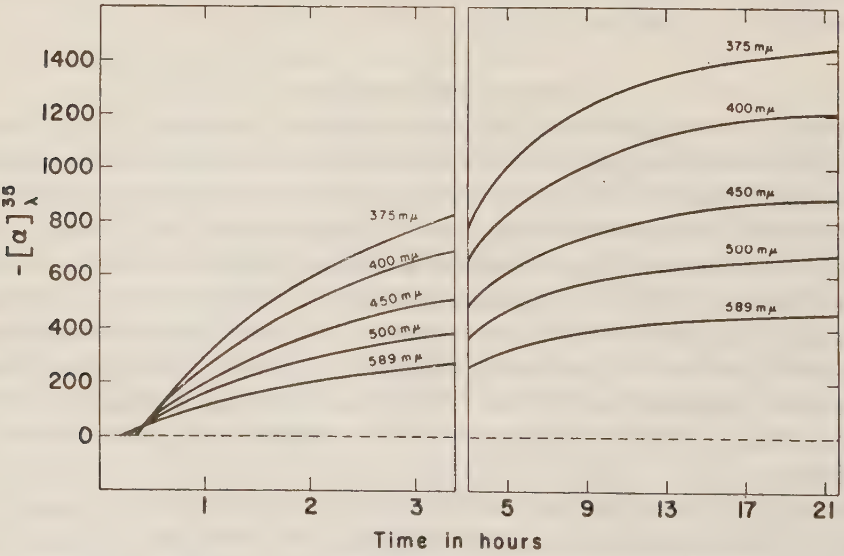

Figure 2
The forward mutarotation of poly-L-proline (B 4, molecular weight 18,000) in glacial acetic acid at 35°, measured at five different wavelengths.

was $-100°$. At the very early stages of the mutarotation the dispersion was anomalous, i.e. it did not obey a one Drude term equation. Anomalous dispersion for polyproline I in acetic acid was reported previously by Blout and Fasman[10]. On the other hand the dispersion of a low molecular weight polyproline in water was normal, i.e. it obeyed a one Drude term equation, through the whole forward mutarotation range[3].

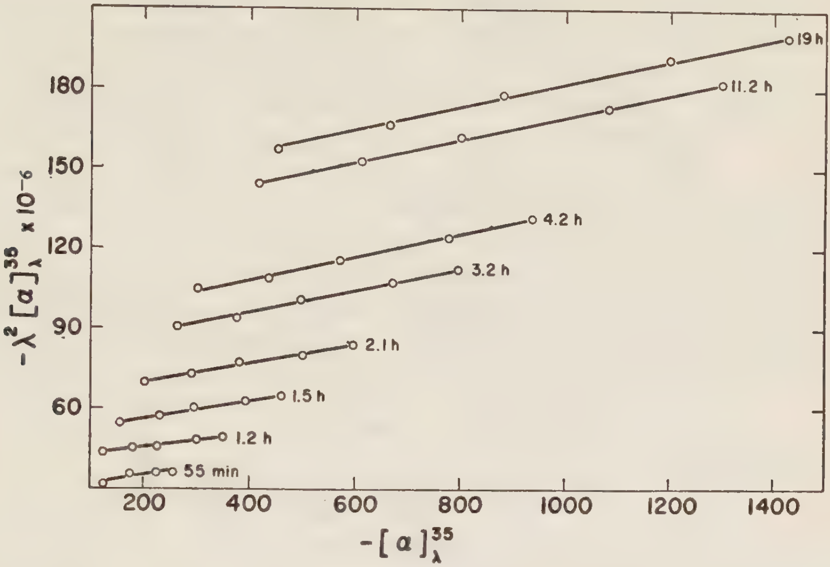

Figure 3
Modified dispersion plots for polyproline at various times of the forward mutarotation in glacial acetic acid at 35°.

Optical rotatory dispersion measurements during the reverse mutarotation of poly-L-proline (B 2, molecular weight 5,000) in formic acid: *n*-butanol (1:9 v/v) are presented in Figure 4. In this solvent mixture polyproline I has a negative rotation in the whole wavelength range investigated ($[\alpha]_D = -20°$). Anomalous dispersion was also found in this system in the range of $[\alpha]_D = -100°$ to $[\alpha]_D = -20°$.

From the slopes of the straight lines given in Figure 3, as well as from the data presented in Figure 4 in the range of normal dispersion, it was possible to calculate the critical wavelengths, λ_c (see Figure 5). It should be recalled that when the optical rotatory dispersion obeys a single Drude term equation, i.e. when it can be described by:

$$[\alpha]_\lambda = \frac{K}{\lambda^2 - \lambda_c^2}$$

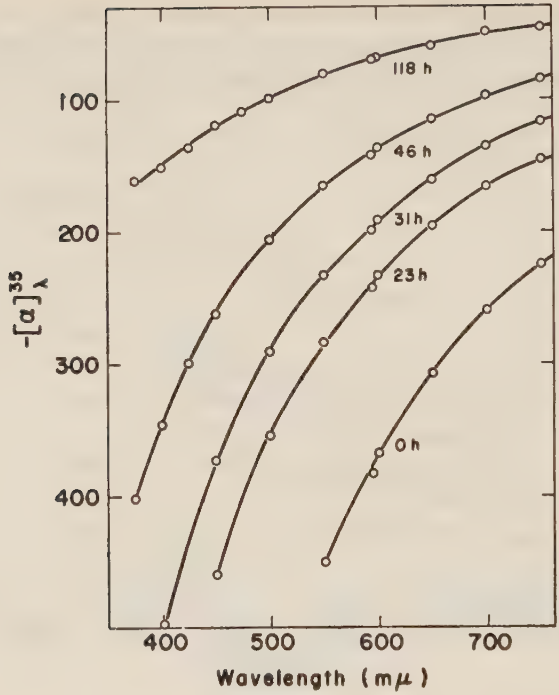

Figure 4
Optical rotatory dispersion of poly-L-proline (B 2, molecular weight 5,000) during the mutarotation from Form II to Form I, in formic acid: *n*-butanol (1:9 v/v) at 20°.

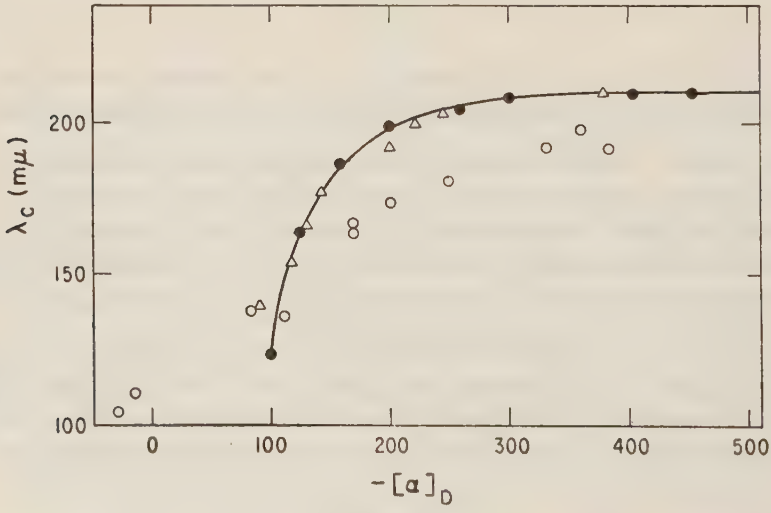

Figure 5
The rotatory dispersion constant λ_c, of poly-L-proline as a function of the specific rotation, $[\alpha]_D$ ●——● data taken during forward mutarotation in glacial acetic acid; △——△— data taken during reverse mutarotation in formic acid: *n*-butanol (1:9 v/v). For comparison, data taken (Harrington and Sela[3]) during forward mutarotation in water (○——○) are included.

a plot of $\lambda^2[\alpha]_\lambda$ versus $[\alpha]_\lambda$ should give a straight line with a slope of λ^2_c. K and λ_c are characteristic constants.

In Figure 5 the λ_c values calculated are given as a function of the extent of mutarotation as expressed by $[\alpha]_D$. The relation between λ_c and $[\alpha]_D$ is the same for the forward and reverse mutarotation, both conducted in nonaqueous media. This relation differs, however, from that reported previously[3] for the forward mutarotation in water. It might be noted that the water-soluble sample of poly-L-proline I used by Harrington and Sela showed normal dispersion in aqueous solution at all stages of the mutarotation. The same sample when investigated in propionic acid exhibited anomalous rotatory dispersion.

The viscosity of poly-L-proline in Form I is always less than that of Form II in a given solvent at the same concentration. Forward and reverse mutarotations are, therefore, necessarily accompanied by changes in viscosity. As expected, the viscosity increases in a monotonic fashion during the forward mutarotation in glacial acetic acid (Figure 6). The viscosity changes during the reverse mutarotation in acetic acid: *n*-propanol (1:9 v/v) are also recorded in Figure 6. The initial viscosity in this solvent system is lower than that of the same substance in acetic acid. The viscosity decreases with time in a monotonic fashion; it increases, however, on short heating to complete the reaction.

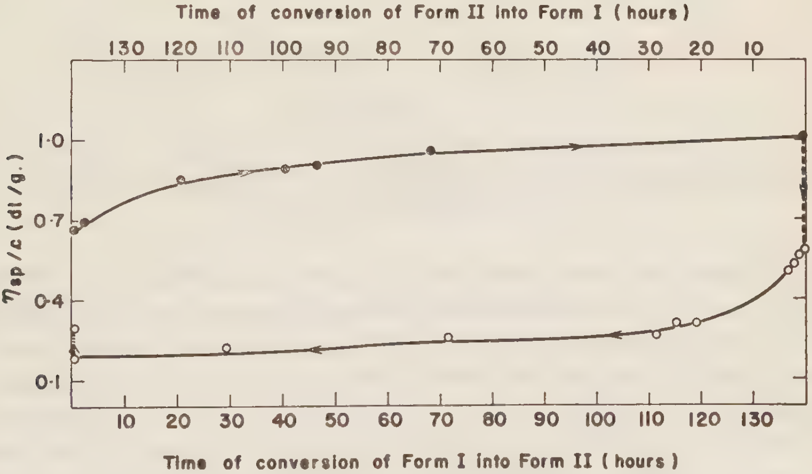

Figure 6

Reduced viscosity of poly-L-proline (c = 1.0 g per 100 ml) at 30°. ●—●-during the conversion of Form I into Form II, in glacial acetic acid; ○—○-during the conversion of Form II into Form I, in acetic acid: *n*-propanol (1:9 v/v).

The viscosity changes during the forward mutarotation in acetic acid-water (Figure 7) took an entirely different course from that recorded in acetic acid alone. During forward mutarotation in acetic acid-water (7:3 v/v, at 30°) the viscosity decreases at first, passes through a minimum and increases during the later stages of the reaction. A similar viscosity minimum was observed previously[3] during the forward mutarotation of a low molecular weight sample of poly-L-proline in water.

From the above it seems that the course of the conversion of polyproline I into polyproline II is determined by the nature of the solvent in which it takes place.

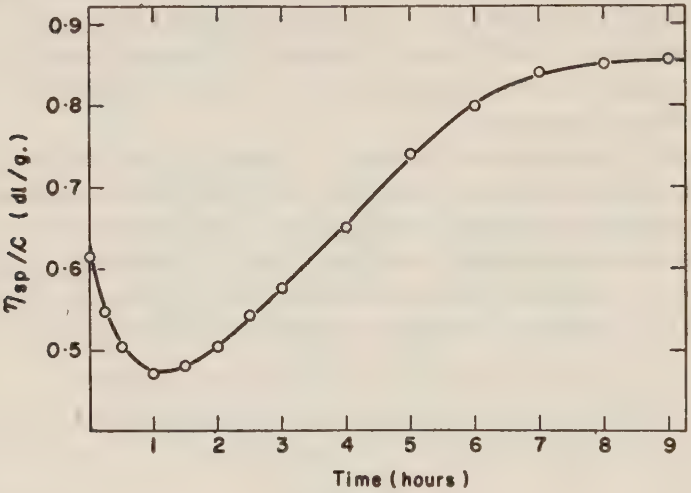

Figure 7
Reduced viscosity of poly-L-proline (C = 1.0 g. per 100 ml) at 30° during the forward mutarotation in acetic acid-water (7:3 v/v).

In the presence of water the optical rotatory dispersion is normal in the whole mutarotation range, the λ_c versus $[\alpha]$ plot is different from that in glacial acetic acid, the viscosity passes through a minimum, and the mutarotation follows a zero order kinetics. In glacial acetic acid[4] the mutarotation could be described by kinetics of the order 4/3.

The changes in viscosity upon mutarotation are plotted versus $[\alpha]_D$ in Figure 8. The curves were obtained from viscosity and mutarotation kinetics during the interconversion of the two forms of polyproline. It should be noted that at the first stages of the forward mutarotation in acetic acid marked changes in specific rotation are accompanied by a rather small increase in viscosity. On the other hand, at the later stages of this mutarotation, when the reaction is practically complete, the viscosity continues to increase to its final value. This can also be observed in the two other cases recorded in Figure 8.

In view of the above it can be concluded that while the viscosity is determined primarily by the end-to-end distance of the polymer, the optical rotation is a reflection of the helical structure of relatively short segments. These considerations demonstrate that the factors determining hydrodynamic properties on the one hand, and optical rotatory properties on the other hand, allow a given molecular species to take up

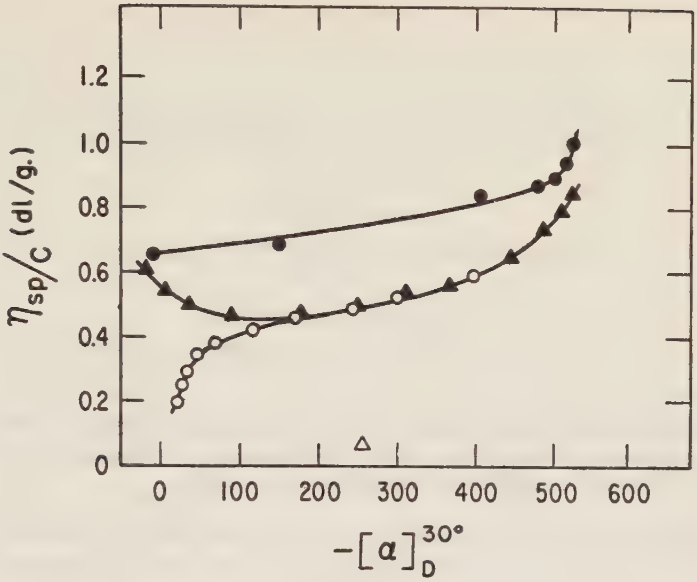

Figure 8
Reduced viscosity (c = 1.0 g per 100 ml) at 30° as a function of $[\alpha]_D^{30}$. ●——●-data taken during forward mutarotation in glacial acetic acid; ▲——▲-data taken during forward mutarotation in acetic acid-water (7:3 v/v); ○——○-data taken during reverse mutarotation in acetic acid-*n*-propanol (1:9 v/v); △——△-value in aqueous 12 M LiBr.

different shapes as measured by viscosity but still possess essentially identical rotatory properties. This situation is clearly seen in Figure 8.

ACKNOWLEDGEMENT

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HELIX-COIL TRANSITION OF POLY-L-GLUTAMIC ACID IN A CHLOROETHANOL — WATER MIXTURE

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ABSTRACT

The helix-random coil transition of poly-L-glutamic acid in a 20% 2-chloroethanol–water mixture was investigated. This transition is shown to be a function of the ionisation, and to take place at a degree of neutralisation of about 0.45.

Poly-α-amino acids[1] were recently shown to possess in the solid state[2] and in solution[3,4] a secondary structure similar to that of proteins[5,6]. In view of the considerable amount of data now available on the electrochemistry and configurational changes of synthetic polyelectrolytes[7,8], it seemed of interest to study the change in configuration as a function of the degree of ionisation (α) of poly-α-amino acids possessing a large number of ionisable side chains.

Poly-L-glutamic acid (PGA) was chosen as the model compound in the following investigation. The helical and random configurations of poly-γ-benzyl-L-glutamate have been extensively investigated[3,4]. The configurational analysis of the corresponding free polyacid in solution seems, however, to be merely at its beginning[9,10].

The electrochemical, viscosity and optical rotatory properties of poly-L-glutamic acid reported in the present study were determined in a 2-chloroethanol–water mixture (2:8 v/v). This solvent was chosen since it dissolves the free poly-L-glutamic acid, as well as its sodium salt.

The experimental data indicate that under the conditions used, the synthetic polypeptide retains an α-helix structure up to a degree of neutralisation of $\alpha = 0.45$ and a random coil configutation at $\alpha > 0.55$, the helix-coil transition taking place between $\alpha = 0.45$ to 0.55.

EXPERIMENTAL

Materials

Poly-γ-benzyl-glutamate was prepared according to Blout and Karlson[11]. The degree of polymerization, DP = 755, was determined viscometrically in chloroform saturated with formamide[12].

Poly-L-glutamic acid (PGA) was prepared by debenzylation of poly-γ-benzyl-L-

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glutamate in acetic acid–hydrogen bromide mixture[15,16] at 4°C. The PGA thus obtained was washed with ether and dried in vacuo.

The sodium salt of polyglutamic acid was prepared by the neutralisation of a PGA solution in dimethyl-formamide with an equivalent quantity of sodium methoxide. The polymer salt was precipitated with anhydrous ether, washed with ether and absolute methanol (to remove traces of unreacted sodium methoxide) and dried in vacuo. The dry material was finally dissolved in a minimum amount of water and lyophilized.

The degree of neutralisation of the sodium salt of poly-L-glutamic acid obtained (hereafter designated Sample SGl) was 70% (by sodium analysis and direct titration in water to pH 7).

Sample SGl gave a sedimentation constant of $S_{20} = 2.9 \times 10^{-13}$ (pH 6.5, ionic strength 0.5) and a diffusion constant of $D_{20} = 2.0 \times 10^{-7}$ in the same solvent. From these values an average molecular weight of 104,000 was calculated.

2-Chloroethanol (reagent grade) was purified by drying over anhydrous sodium sulphate, shaking with anhydrous aluminium oxide, distillation at normal pressure, and shaking again with aluminium oxide to remove free hydrogen chloride.

The ionic strength of the water — chloroethanol mixtures was usually adjusted with rubidium chloride (reagent grade). Because of its low solubility in the solvent mixture used, potassium chloride was found unsuitable.

Methods

Potentiometric titrations were carried out with a "Photovolt" Direct Reading pH-meter, using calomel and Beckman glass (general purpose) electrodes at room temperature (~ 20°C). The pH-meter was standardized with pH 4.0 and 7.0 standard buffers.

The titrations were carried out on 20 ml solutions containing about 0.25 g polymer (SGl), previously adjusted to acid pH (pH 2.2).

Sodium hydroxide (1N) withdrawn from an "Agla" microburette was used as titrant.

The titration curves were corrected for hydrogen ion uptake of solvent by subtracting the solvent titration curve from that of the polymer solution. Correction for the small volume change due to addition of titrant was not made.

The pH readings were assumed to have the same meaning as in water[14].

Viscosity measurements were made in an Oswald-Fenske viscometer thermostated at 30 ± 0.1°C.

Optical rotation measurements were made with a Rudolph Photoelectric Polarimeter (Model 200). The rotations given were obtained at a wave length of 589 mμ (Sodium D-line).

RESULTS AND DISCUSSION

The potentiometric titration of poly-L-glutamic acid in 20% 2-chloroethanol —

water mixture (volume per volume) at an ionic strength of 0.1 in rubidium chloride is given in Figure 1. The experimental curve shows a distinct break in its slope at about *p*H 5.8, corresponding to a degree of neutralisation of $\alpha \cong 0.45$.

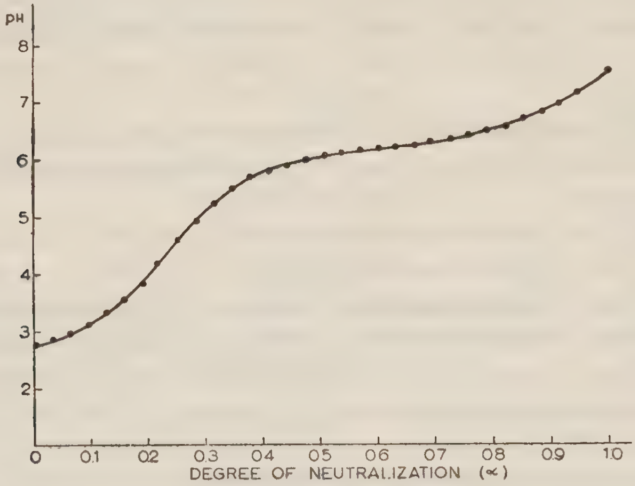

Figure 1
Potentiometric titration of poly-L-glutamic acid in 20% chloroethanol–water mixture at ionic strength 0.1 in RbCl at room temperature (~20°C).

The reduced specific viscosity ($\eta_{SP/C}$ at $c = 0.25$ g/100 ml) as a function of the degree of neutralisation in the same solvent system is recorded in Figure 2. The experimental curve has a minimum at $\alpha \cong 0.57$.

The change in specific optical rotation with ionisation is presented in Figure 3. The steep decrease in the optical rotation takes place at *p*H $\cong$ 5.8, corresponding to an $\alpha \cong 0.45$.

It has been shown that the transition from a left-handed alpha-helix configuration[3-6,13] to a random coil in proteins and poly-α-amino acids is accompanied by a large decrease in optical rotation.

The marked decrease in $[\alpha]_D$ at about *p*H 5.8 from $[\alpha]_D = +7°$ to $[\alpha]_D = -75°$ seems to indicate, therefore, that the α-helix configuration collapses when the degree of neutralisation reaches a value of $\alpha \cong 0.5$.

The disruption of the asymmetric macromolecular configuration undoubtedly results from the strong intramolecular repulsive forces on the one hand, and the breakdown with ionisation of the hydrogen bonds between adjacent carboxyls which seem to stabilize the helix structure.

The helix-coil transition indicated by the change in optical rotation should be accompanied by the appropriate change in viscosity. The relatively high viscosity of the poly-L-glutamic acid solution at low degrees of neutralisation ($\alpha = 0$ to 0.2)

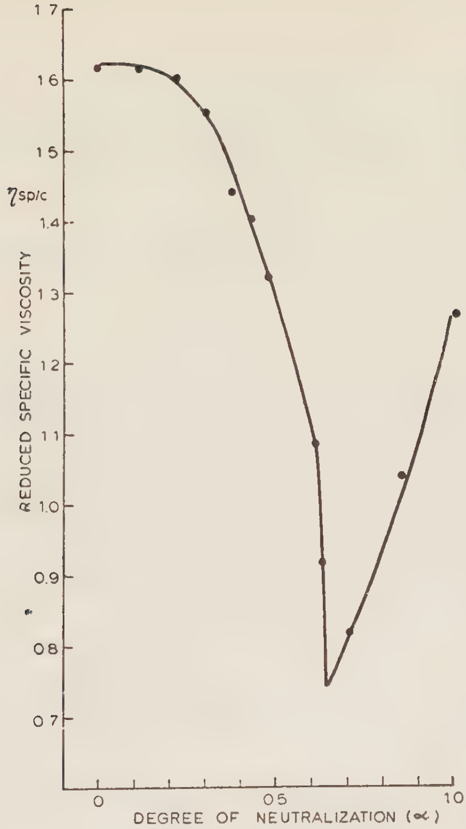

Figure 2

Reduced specific viscosity $\eta_{SP/C}$ vs. degree of neutralization (α) of poly-L-glutamic acid in 20% chloroethanol–water mixture, ionic strength 0.1 in RbCl, temperature 30 ± 0.1°C, concentration of solution 0.25 gm/100 ml.

implies high asymmetry for the dissolved macromolecules, in accord with the assumed structure. In the range of $\alpha = 0.2$ to 0.6, ionisation increases the flexibility of the helical rod, and thus leads to a decrease in viscosity.

Complete destruction of the helical regions as a result of further charging transforms the polyglutamic aicd to a randomly coiled polyelectrolyte, whose viscosity increases with charge[7,8].

Further support in favour of the above interpretation is supplied by the observation that the viscosity is strongly ionic-strength-dependent, particularly in the random coil region.

The break in slope of the potentiometric titration reveals a change in electrostatic potential of the polyacidic peptide molecule, probably due to the configurational changes discussed.

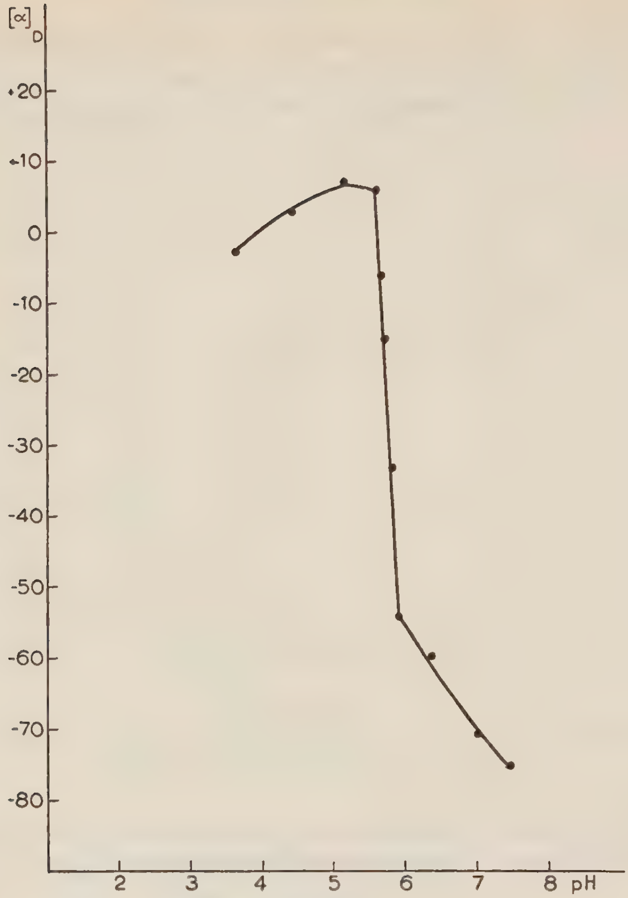

Figure 3

Specific rotation $[\alpha]_D$ vs. pH of poly-L-glutamic acid in 20% chloroethanol–water mixture, ionic strength 0.1 in RbCl, at room temperature.

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WATER SOLUBLE FORAGE CONSTITUENTS AND THEIR INFLUENCE ON THE METABOLIC ACTIVITY OF THE RUMEN*

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ABSTRACT

The contents of monosaccharides, disaccharides and fructosans and of non-protein nitrogen compounds (free amino acids, peptides and amides) were determined in 5 different samples of green fodder and hay: Alexandrian clover cut at 2 different stages of growth, rye grass, Alexandrian clover hay and rye grass hay. Rumen liquor was taken from sheep which were fed exclusively on one of these 5 forages and the metabolic activity of the rumen liquor was determined; standard feeds as clover meal and soybean protein were incubated with the different samples of rumen liquor. The extent of cellulose digestion and the amount of ammonia liberated served as an index of the metabolic activity of the rumen.

A linear relationship between the extent of decomposition of cellulose and the content of soluble carbohydrates and the non-protein nitrogen compounds contained in the feeds was established.

No correlation between the extent of cellulose digestion and the deaminating capacity of the rumen liquor could be found.

Rumen liquor taken from sheep which were fed on hay considerably surpassed the deaminating capacity of rumen liquor obtained from sheep fed green forages.

The nutritive value of non-protein nitrogen compounds and sugars contained in forages has been disregarded by the classical methods of feed estimation and therefore very few investigations on these food constituents have been carried out.[1] The decisive role played by microbial processes — such as the breakdown of cellulose and the attack of proteins — in the nutrition of ruminant animals is now recognized unequivocally[2]. Generally, soluble compounds of low molecular weight are easily utilized by micro-organisms as judged from stimulation of their metabolism. It is therefore the aim of this paper to prove the existence of a correlation between the content of sugars and non-protein nitrogen compounds in forages and the utilization of forages by ruminant animals. Standard feeds were incubated with rumen liquor obtained from sheep fed exclusively on forages of known low-molecular compound content. The extent of cellulose digestion and the amount of ammonia liberated in these *in vitro* experiments served as an index of the metabolic activity of the rumen. The evolution of ammonia in the rumen precedes the conversion of food protein into microbial protein which is utilized by the host animal similarly to dietary protein

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* This paper is dedicated to the memory of the late Dr. Hanan Meyer, who was associated with the senior author in research on animal nutrition for a period of 14 years (1937–51).

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by monogastric animals. Therefore, the extent of ammonia formation — up to a certain limit — reflects the activity of the rumen micro-organisms. The validity of such *in vitro* studies with rumen micro-organisms has been established by recent research[3] and the results of these studies can be applied to practical problems in animal nutrition.

EXPERIMENTAL

Analytical determination of simple carbodyhrates and non-protein nitrogen compounds in forages

Alcoholic extract: 25 g fresh plants (minced) or 10 g ground hay were extracted in 4 portions with 500 ml alcohol (80% v/v) in a Waring blendor — each portion was blended for 5–7 min. (In the case of hay it is recommended to soak the sample with alcohol for a few hours before using the Waring blendor). The combined solutions were filtered through a Buchner funnel, evaporated under reduced pressure at a temperature below 50° C to about 50 ml and finally diluted with water to 100 ml. Plant pigments were extracted with ether and the aqueous solution freed from dissolved ether by heating in a hot water bath. This solution served for the following determinations: *mono-saccharides* and *disaccharides* (after hydrolysis by HCl) according to van der Plank[4]; *amino acids* by the copper-titration of Pope and Stevens[5]; and *amides* according to Vickery *et al*[6]. (Acidifying 10 ml solution by adding H_2SO_4 to 1N concentration, hydrolysis in boiling water for 2 hrs and ammonia determination according to Conway[7] in 0.5–2 ml solution).

Water extract: The residue after alcoholic extraction was treated in the Waring blendor with 400 ml water for 5–7 min. The solution was cleared by centrifugation. After precipitation of proteins by glacial acetic acid, the solution was centrifuged again and evaporated at reduced pressure to 50 ml. Amino acids (non-soluble in 80% alcohol) and peptides were determined in this solution.

Peptides were precipitated by addition of alcohol to 75% concentration. The precipitate was separated by centrifugation and hydrolysed under reflux (8 hrs. by 5 N HCl). Amino-nitrogen was determined before and after hydrolysis (Ferguson and Terry)[8].

Fructosans were determined in a hot water extract according to de Man and de Heus[9]. For this purpose the residue of the cold water extraction was refluxed with 200 ml. water and the filtered solution evaporated under reduced pressure to 80 ml, after which fructosans were determined.

Rumen liquor was taken from about 5-month old rams, which were fed exclusively on one of the 5 kinds of green fodder or hay for at least 10 days (see Table I). These forages and drinking water were allowed *ad libitum*. The sheep used at the beginning of this work were fitted with a permanent rumen fistula; later the rumen liquor was removed by stomach tube from the sheep.

Incubation of soybean-protein with rumen liquor. Liberation of NH_3 was measured by the following procedure which was developed after the method described by

El-Shazly[10]. Ten ml rumen liquor were added to 100 mg soybean-protein (Bios Lab., N.Y.) in a test tube (20 × 100 mm) and covered by 1 ml liquid paraffin. Control experiments were carried out simultaneously with omission of the protein. The mixtures were incubated for 4 hrs. at 38°C and were mixed thoroughly at the beginning of the experiment and every half hour during the incubation. Ammonia was determined in 1 ml samples of experiment and control; the difference of both results corresponds to the amount of ammonia liberated by the rumen activity from soybean protein.

The extent of *cellulose digestion by rumen liquor* was measured according to Huhtanen, Saunders and Gall[11]; 500 mg clover meal served as substrate. The non-digested part of the clover meal was separated by centrifugation and hydrolysed by 60% (v/v) H_2SO_4 and the glucose contained in this solution was determined by Roe's anthron-method[12]. The cellulose content of the clover meal was determined as well.

RESULTS AND DISCUSSION

The contents of soluble carbohydrates and non-protein nitrogen compounds of 5 forage samples fed to sheep are presented in Table I. The samples of 3 different kinds of green forages contain similar amounts of soluble carbohydrates. A comparison, however, of the amounts of soluble constituents in both clover samples cut at different ages reveals the general trend of decrease during plant growth. The consider-

TABLE I

Contents of soluble carbohydrates and Non-Protein Nitrogen compounds of forages

Feed constituent in % of dry matter	Alexandrian clover, green, cut at 3 weeks	Alexandrian clover, green, cut at 5 weeks	Rye grass green, cut at 2 months	Rye grass hay	Alexandrian clover hay
Monosaccharides	3.70	3.04	2.41	3.36	2.04
Disaccharides	2.27	2.31	2.41	0.52	0.48
Fructosans			0.39	0.73	
Soluble carbohydrates, total	5.97	5.35	5.21	4.61	2.52
Amino N	0.26	0.18	0.37	0.19	0.27
Peptide N	0.11	0.08	0.06	0.02	0.02
Amide N	0.10	0.05	0.04	0.04	0.04
Non-protein nitrogen (N.P.N.)	0.47	0.31	0.47	0.25	0.33
N.P.N. × 6.25	2.94	1.94	2.94	1.56	2.06
Sol. carbohydrates + N.P.N.	6.44	5.66	5.68	4.86	2.85
Sol. carbohydrates + N.P.N. × 6.25	8.91	7.29	8.15	6.17	4.58
Total N	3.87	3.24	2.14	1.94	2.94
N × 6.25	24.2	18.9	13.4	12.1	18.4

able decrease of sugar content occurring during hay-making is remarkable, especially the loss of disaccharides which were apparently transformed first to mono-sugars. A considerable part of the mono-sugars disappeared afterwards, possibly as a result of respiration and fermentation. A similar decrease in the content of the small peptide fraction was observed during the transformation of green fodder to hay. It seems that hydrolysis of both peptides and proteins takse place during hay-making; a part of the liberated amino acids disappeared as a result of leaching and degradation.

The influence of soluble compounds present in the diet of sheep on the activity of rumen liquor was examined by different experiments and their results are summarized in Table II.

TABLE II

The influence of the diet on the in vitro digestion of cellulose and protein by rumen liquor

Diet of sheep	Clover, green cut at 3 wks	Clover, green cut at 5 wks	Rye grass green	Rye grass hay	Clover hay
% of cellulose (contained in clover meal) digested	70	57	60	59	52
NH_3-N mg liberated from 100 mg soybean	9.25	4.98	2.78	11.63	11.43

All the values are average results of 6 experiments, carried out with samples of rumen liquor taken from each of 2 sheep on 3 different days. A clear correlation was found between the extent of the cellulolytic power of the rumen and the content of soluble compounds in the diet provided to sheep before the sampling of rumen liquor. This parallelism can be observed if the amount of soluble carbohydrates *per se* or the sum of soluble carbohydrates + non-protein nitrogen contained in feed are considered as criteria for judging the influence of the diet on the cellulolytic power of the rumen This correlation appears from the curves presented in Figure 1 and 2. The data of curve 2 were calculated by adding the sum of carbohydrates and the N-values of the 3 non-protein nitrogen (N.P.N.) fractions. N.P.N. values multiplied by 6.25 were used for the respective calculations of the data pertaining to curve 3. The multiplication of N.P.N. values by 6.25 seems to be justified as the N.P.N. compounds are converted into proteins by bacteria. The referred correlation is asserted for both modes of calculation. These curves reveal one deviation only. Although the content of soluble carbohydrates of adult clover surpasses the respective percentage in fresh rye grass, the rumen liquor withdrawn from rye grass fed sheep showed a cellulolytic power even higher than that obtained after feeding adult clover. It may be considered that the content of soluble sugars or of simple soluble compounds is not the sole dietetic factor controlling the extent of cellulose digestion. The fructosans present only in rye grass or the high free amino-acid content of rye grass may have contributed to the rise in cellulolytic power.

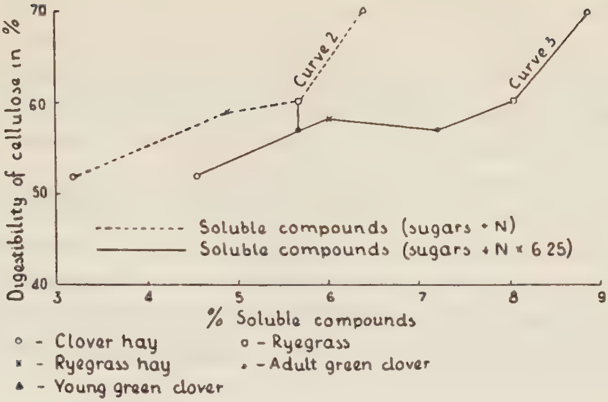

Figure 1
Correlation between content of forages and cellulose digestion *in vitro*

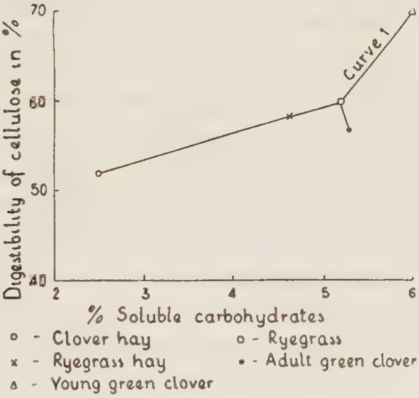

Figure 2
Correlation between content of soluble compounds in forages and cellulose digestion in vitro

No correlation between the protein content of the ration and the cellulolytic power of the rumen, which would be logically expected, could be established at all. This feature agrees with experimental results of Head[13] — that increasing the amount of protein-nitrogen above approximately 1% of the total ration of sheep does not affect the digestibility of cellulose. No interrelationship between cellulolytic and deaminating power of the rumen liquor could be found either.

The great difference observed in the deaminating power of rumen liquor of sheep fed on hay or green forages is, however, most striking. Although NH_3 concentrations in rumen liquor of sheep fed hay or green fodder did not originally differ, the deaminating power of the rumen liquor obtained from hay-fed sheep was considerably higher. Differences in the chemical composition of green forages and hay cannot provide a probable explanation for this phenomenon. It may be assumed that the physical structure of dried forages enables the perfusion of micro-organisms and therefore allows for a better utilization of high nutritive constituents of hay, which may lead to greater growth of bacteria of high deaminating capacity. If the 3 kinds of fresh forages are considered separately, a correlation can be established between the deaminating power of rumen liquor and the contents of soluble compounds, of amides and of total nitrogen in these forages (see Tables I, II).

Practical feeding systems for ruminant animals differ by the ratio of fresh to dried forages or by the use of comparatively young plants (soilage) or more adult, somewhat woody plants (cultivated pasture). Therefore systematic investigations on the influence of these different forages on the rumen metabolism, which are under way in this department, may lead us to a more economic composition of the feed ration than is used today.

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A *p*H-STATIC METHOD FOR THE ASSAY OF ARGINASE

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ABSTRACT

As the δ-amino group of ornithine is less basic than the guanido group of arginine and urea is an extremely weak base, arginase activity is accompanied by the release of hydrogen ions. This fact forms the basis of a *p*H-static method for assaying arginase, by the rate of addition of alkali in the presence of a large amount of substrate; alternatively, the *p*H-static method may be used for the determination of small amounts of L-arginine by measuring the amount of alkali which must be added until hydrolysis is complete. Potentiometric titration curves and consideration of the activity of the enzyme indicate that the sensitivity of the method is greatest near *p*H 10. A "*p*H 10.2 arginase unit" is proposed for the assay, which is defined as the amount that in 1 minute at 25°C and *p*H 10.2, at a substrate concentration of 0.285 M will decompose 1 μmole of arginine. At this *p*H and temperature 1 μmole of alkali added *p*H-statically is equivalent to 2.5 μmoles of L-arginine hydrolysed. A procedure for the assay is given.

As the δ-amino group of ornithine is less basic than the guanido group of arginine and urea is an extremely weak base, the cleavage of L-arginine by arginase is concomitant with the release of hydrogen ions. This fact forms the basis of a *p*H-static method for the assay of arginase, to be described below.

The method consists in measuring the rate of addition of alkali to a weakly-buffered arginase system in the presence of a large amount of arginine. Though non-specific, the method is rapid and convenient, and enables one to follow the course of the reaction. The *p*H-static method may be used to assay arginase by the *rate* of addition of alkali, and also to determine small amounts of L-arginine by the *amount* of alkali added to an arginase system until hydrolysis of the substrate is complete.

The selection of a suitable *p*H for the *p*H-static assay of arginase must necessarily be governed by two factors: (a) the *p*H-range of arginase activity, which is generally near *p*H 10; (b) the *p*H that would require the addition of the greatest amount of alkali for a given amount of arginine, that is, the *p*H at which the assay would be most sensitive.

In order to find a sensitive *p*H range, potentiometric titrations were carried out on an arginine solution and a solution containing ornithine and urea in equivalent amounts (Figure 1). The quantity of alkali represented in the figure by the horizontal segment *a*, divided by the quantity of arginine titrated (400 μmoles), gives the ratio of alkali that should be added *p*H-statically to a given amount of arginine if hydrolysed completely to ornithine and urea. This ratio will be referred to hereafter as the alkali/arginine ratio.

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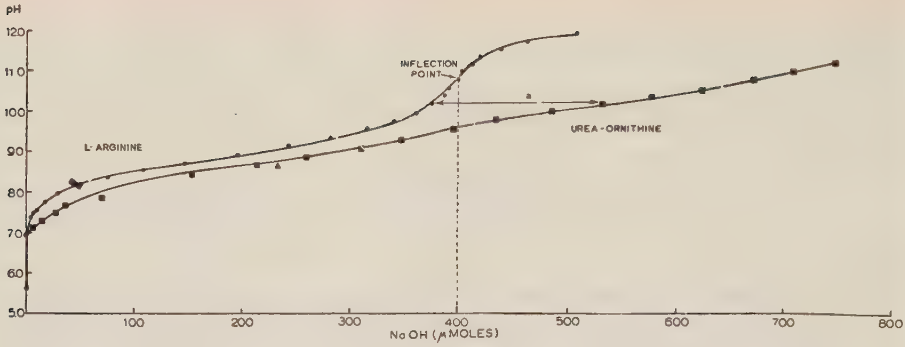

Figure 1
Potentimetric titration curves
Arginine solution, 400 μmoles in 5 ml. Urea-ornithine solution, 400 μmoles of each in 5 ml. Titrant, 1 N NaOH. Titrations were carried out at room temperature.

It can be seen from the curves that the sensitivity of the assay would be small below pH 9.5; on the other hand, above pH 10.2, though the curves continue to diverge, the assay would hardly be feasible because arginase activity dropes sharply[1]

The titration curves led to the investigation of the possibility of assaying arginase pH-statically at pH 9.5 (the pH used by Van Slyke and Archibald in defining arginase activity[2]) and at pH 10.2. As read from the curves, the alkali/arginine ratio at these pH would be 0.221 and 0.387 respectively.

APPARATUS AND MATERIALS

A Radiometer titrator type TTTla and recorder type SBRlb with microburette unit type SBUl and Agla micrometer-syringe were used. The reaction-vessel was conical on the inside, with a water jacket through which water was circulated from a thermostatic water-bath by means of a pump. The vessel was provided with a magnetic stirrer and fitted with a rubber stopper with soda-lime cap, through which the shielded glass electrode, agar bridge to the calomel electrode, delivery tube of the Agla micrometer syringe, inlet and outlet tubes for the passage of nitrogen were passed.*

L-Arginine-hydrochloride from Nutritional Biochemicals Corporation and, unless otherwise stated, arginase from Worthington Biochemical Corporation were used.

RESULTS AND DISCUSSION

*p*H-*static determination of the alkali-arginine ratio*

In the pH-static determination of the alkali/arginine ratio, the action of arginase on known amounts of L-arginine was followed on the pH-stat up to the end of the reaction. The procedure was as follows: 2.5 ml of a solution containing 1–2 mg per ml of arginase in 8 × 10^{-4} M $MnSO_4$ and 1.76 per cent NaCl (to prevent precipitation of the enzyme) were added to 2.5 ml of an arginine solution. Alkali (0.1 to 0.5 N NaOH) was added *p*H-statically until no more was required for at least 10 minutes. The net amount of alkali required was the total read on the graph, minus the amount required to bring the arginase solution to the desired *p*H. Blanks run separately for the arginine and arginase solutions showed a negligible alkali consumption versus time.

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* The same assembly may be used equally well with a well–earthed Beckman Model G pH-meter.

Typical curves, showing the course of the reaction at *p*H 10.2 and 37° are shown in Figure 2. As was to be expected, and as is shown by the straight line in Figure 3, the alkali/arginine ratio is a constant. At *p*H 10.2 and 37° this ratio is 0.583, with a standard deviation for the points on the line of 0.025.

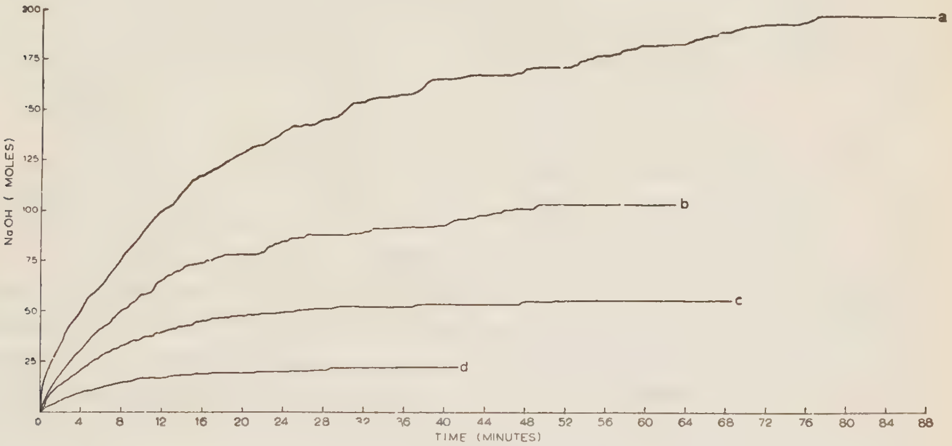

Figure 2
Course of the arginase reaction (pH 10.2 and 37°)
Composition of reaction mixture (5 ml): Arginine, μmoles (a) 320; (b) 160; (c) 80; (d) 32. Arginase, 0.5 mg/ml. Mn^{++}, 5x10^{-4} M NaCl, 0.88%. Titrant, 0.5N NaOH.

Similar determinations of the alkali/arginine ratio were carried out *p*H-statically at *p*H 10.2 and 25° and at *p*H 9.5 at 37° and 25°. Here the determinations were made in triplicate on a single arginine concentration and the results were averaged. The runs at 25°

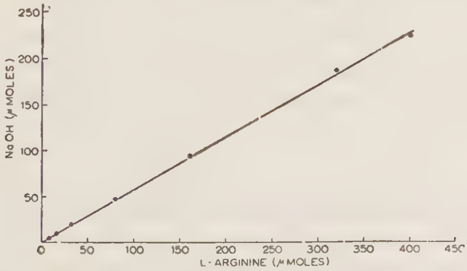

Figure 3
The ratio alkali to arginine (pH 10.2 and 37°)

(both *p*H 9.5 and 10.2) were carried out in conditions simulating those required by Greenberg[3] for the assay of arginase, namely a 3–4 hour preincubation of the enzyme in 0.05 M $MnSO_4$–0.05 M maleate (the latter being added to prevent the precipitation of Mn^{++} at *p*H 7.0)*. The results of these determinations, summarized in the accompanying table, show that the alkali/arginine ratios determined *p*H-statically at 25° are in very good agreement with the ratios abtained from the titration curves (0.221 at *p*H 9.5 and 0.387 at *p*H 10.2).

TABLE I

The alkali/arginine ratio determined p*H-statically*

*p*H	°C	Arginase mg/ml	Arginine μmoles (per 5 ml of reaction mixtures)	alkali/arginine ratio
9.5	25	0.5	200	0.220
	37	1.0	250	0.335
10.2	25	0.83	200	0.394
	37	0.5	8–400	0.583

The alkali/arginine ratio at *p*H 10.2 is higher than at *p*H 9.5, while the buffer capacity of arginine is lower (Figure 1). Therefore a *p*H-static method for the assay of arginase would be more sensitive at the former *p*H. It would likewise be more sensitive at 37° than at 25°. However, in the assay of arginase to be described below, a temperature of 25° is suggested, as at this temperature the method was both sensitive and convenient. As can be seen from the table, at *p*H 10.2 and 25°, 1 mole of alkali is practically equivalent to 2.5 moles of arginine.

Another series of experiments showed that the source or concentration of the arginase had no effect on the amount of alkali consumed up to the end of the reaction, i.e. on the alkali/arginine ratio. This is so inasmuch as the concentration of enzyme is high enough to split the arginine at a sufficiently rapid rate. Owing to the instability of the enzyme above *p*H 9.0, the small quantity of enzyme present at low concentrations is dest-royed before the reaction is complete. In one experiment, each of the following enzyme preparations was used on 5 ml of a 0.048 M solution of L-arginine: (a) the purified enzyme, final concentration 1 mg per ml; (b) the supernatant obtained by centrifugation of a rat liver homogenlate in ten times its volume of ice-cold water[4]; (c) a 1:5 dilution in water of the rat liver preparation; (d) the supernatant of a rat kidney homogenate obtained as in

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* It was found that if the Mn^{++} concentration in 5 ml of the reaction mixture does not exceed 5×10^{-4} M, the presence of Mn^{++} ions does not necessitate the addition of alkali during the reaction (*p*H 10.2). Mn^{++} concentrations between 5×10^{-4} and 5×10^{-3} make it necessary to add alkali, probably owing to the removal of OH^- ions as $Mn(OH)_2$. The amount of alkali, however, can be accounted for by running a blank. Final Mn^{++} concentrations above 5×10^{-3}M should be avoided.

(b). In the first three cases the amount of alkali required until completion of the reaction was identical. The kidney preparation acted very slowly and the curve flattened out after nearly two hours at 65 per cent of the expected value. As already explained, the incomplete reaction may be attributed to the low concentration of enzyme. Indeed, when 4 mg of the purified enzyme (Worthington) in 2 ml of water were added to the mixture, the reaction was completed in 15 minutes.

Under the conditions studied, when more than 400 μmoles of arginine are present in 5 ml of mixture, the time required for completion of the reaction is generally about one and a half hours. As the enzyme loses activity above *p*H 9.0, the *p*H-static method appears to be unsuitable for the determination of more than 400 μmoles of arginine. Another factor that detracts from its value as a method for determining arginine is that it is often difficult to decide exactly when the reaction has reached completion, as towards the end of the reaction the course of the graph becomes somewhat irregular. The reason for this may be that at *p*H 10.2 the glass electrode is less sensitive than at lower *p*H.

Assay of arginase

In contradistinction to the *p*H-static determination of arginine, which requires the measurement of the amount of alkali-added until the reaction is complete, the

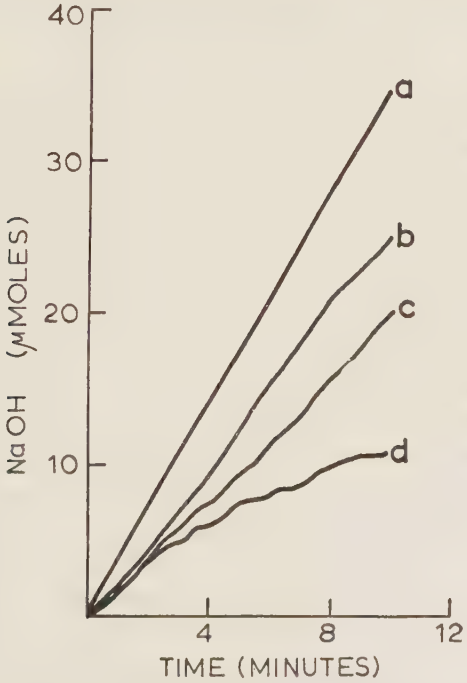

Figure 4

Initial rates of reaction at *p*H 10.2 and different enzyme concentrations

Composition of reaction mixtures (5 ml): Arginine, 0.285 M. Enzyme concentrations, mg/ml: (a) 0.1; (b) 0.075; (c) 0.05; (d) 0.025. Mn^{++}, $5x10^{-4}$ M. Temperature 25^{0}C. Titrant, 0.2 N NaOH.

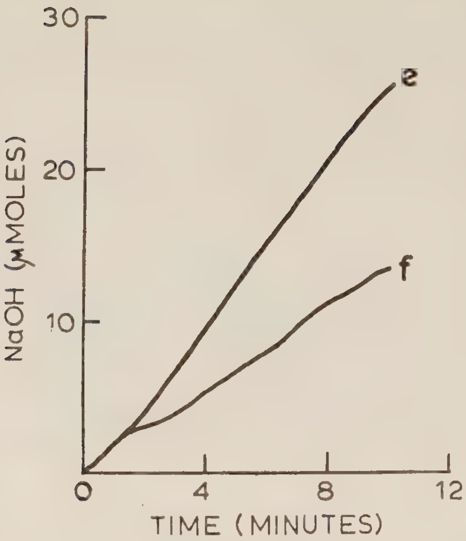

Figure 5
Initial rates of reaction at pH 9.5 and different enzyme concentrations
Composition of reaction mixture (5 ml): Arginine 0.285 M. Enzyme concentrations, mg/ml (e) 0.2 (f) 0.1. Mn^{++}, 5 x 10^{-4}M. Temperature 25°C. Titrant, 0.2 N NaOH.

*p*H-static assay of arginase consists in determining the rate of alkali addition at a high concentration of substrate. This is done, specifically, by measuring the slope of the curve showing alkali addition versus time. At the high substrate concentrations used this rate is generally steady for more than 10 minutes after the beginning of the automatic addition of alkali* and may be measured accurately. Figures 4 and 5 show such initial steady rates in an 0.285 M solution of arginine at various enzyme concentrations at *p*H 10.2 and 9.5. Figure 6 shows the relationship between these rates, measured as the amount of alkali consumed in 10 minutes, and the concentration of enzyme. The relationship is seen to be linear. The three figures show that the method is considerably more sensitive at *p*H 10.2 than at *p*H 9.5.

Van Slyke and Archibald have defined unit arginase activity at *p*H 9.5[2]; it is the amount that in 1 minute at 25° and *p*H 9.5, at a substrate concentration of 0.285 M, will decompose 1 μmole of arginine. Because of the greater sensitivity of the *p*H-static method at *p*H 10.2, *p*H-static assays of arginase are best carried out at this *p*H. Accordingly, a "*p*H 10.2 arginase unit" is here defined as the amount that in 1 minute at 25° and *p*H 10.2, at a substrate concentration of 0.285 M, will decompose 1 μmole of arginine.

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* As arginine acts as a buffer at these high concentrations and at *p*H 9.5 and 10.2, it is impractical to bring the solution to the desired *p*H by means of the Agla micrometer-syringe; it was found preferable to do so by adding 1 N alkali with the aid of a thin-stem 1 ml measuring pipette. In this way the desired *p*H was generally exceeded to an infinitesimal degree, and some time would elapse before the automatic addition of alkali began.

Using the alkali/arginine ratios for 25° C (in table), it can be calculated from Figure 6 that 1 mg of enzyme hydrolysed 16.5 μmoles of arginine per minute at *p*H 10.2 and 11.8 μmoles per minute at *p*H 9.5. Thus the ratio found here between activity at *p*H 10.2 and *p*H 9.5 is nearly 1.4. As the ratio is not in agreement with that obtained from the graph of Mohamed and Greenberg[1], and as it may be affected by several factors, the correlation of "*p*H 10.2 arginase units" with the arginase units of Van Slyke and Archibald is not attempted.

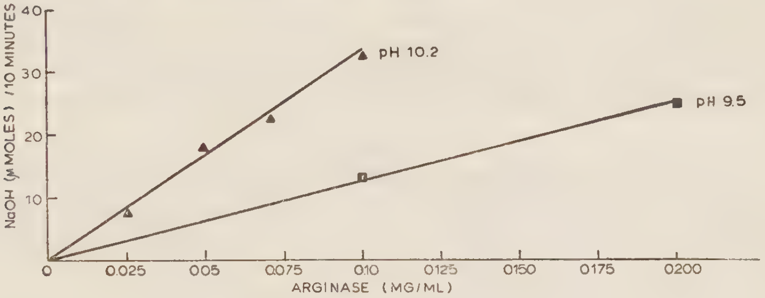

Figure 6
Plot of initial steady rate of reaction versus concentration of arginase at 25^0 C. (for details see Figures 4 and 5).

If, prior to the assay, it is desired to activate the enzyme, this is done by incubating the preparation at 37° for 3 to 4 hours in the presence of 0.05 M $MnSO_4$ and 0.05 M maleate at *p*H 7, and then diluting in water, usually 1:10 or more, so that the final Mn^{++} concentration should be within the limits defined. The enzyme preparation, whether activated and diluted or unactivated, must be of such activity, that when mixed in appropriate volumes with a solution of L-arginine, at a *p*H just under 10.2, it will produce 5 ml of a mixture containing arginine at a 0.285 M concentration that will require at least 0.5 μmoles of alkali per minute (5 μl of 0.1 N NaOH). The use of a 0.57 M solution of L-arginine hydrochloride has been found to be convenient in most cases.

The assay procedure would then be as follows: 2.5 ml of a 0.57 M solution of L-arginine hydrochloride at a *p*H slightly under 10.2 (about 90 per cent of an equivalent amount of alkali is required*) are introduced into the water-jacketed reaction vessel maintained at 25° and connected with the *p*H-stat, recorder, etc. The solution is stirred magnetically, nitrogen is passed over, the *p*H is adjusted to 10.2, the *p*H-stat being set to titrate to this *p*H. 2.5 ml of the suitably diluted enzyme preparation are now added, bringing the arginine concentration to 0.285 M, and the soda-lime tube is placed in the hole that served for the introduction of enzyme. The addition of alkali versus time (using 0.1 N to 0.5 N NaOH) is recorded until a steady slope for

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* This brings the final concentration of NaCl in the reaction mixture to 0.256 M.

10 minutes is obtained. The number of μmoles of alkali consumed per minute, multiplied by the factor 2.5, gives the number of "*p*H 10.2 arginase units" present in the reaction mixture.

The results of the *p*H-static method were compared with those of an assay of arginase at *p*H 10.2 using the colorimetric method of Engel and Engel[5] as modified by Greenberg[3] for estimating urea. The results obtained by the *p*H-static method were roughly 40 per cent higher than those obtained by the colorimetric determination of urea.

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LETTER TO THE EDITOR

Studies on the chemical composition of pollen from *Pinus canariensis*. E. HAVIVI AND J. LEIBOWITZ, *Department of Biological Chemistry, The Hebrew University of Jerusalem.*

The chemical composition of the anemophilous pollen of *Pinus canariensis* was investigated with the purpose of obtaining basic data for a study of the biochemical processes occurring in germinating pollen grains. Pollen was collected in spring 1958 and kept in vacuo at — 20°C until the summer, when the actual analysis was carried out. A general analysis of the pollen is summarized in Table I.

TABLE I

General analysis of Pinus canariensis pollen (per cent of fresh material)

Water	5.8
Ash	2.1
Proteins	7.6
Free amino acids	5.3
N	1.95
Sugars	11.2
Ether–soluble substances	9.8

Free amino acids were isolated by the method of Virtanen and Kari[1] using 250 mg pollen for each analysis. The individual acids were identified with the aid of two dimensional paper chromatography The paper used was Whatman No. 1, the developers were butanol-acetic acid-water (5:1:4) and phenol. The spots were identified after spraying with ninhydrin. Bound amino aicds were determined by acid hydrolysis of the protein according to Marquardt and Vogg,[2] 200 mg pollen were used per analysis. Both ampoule and reflex methods were tried. The hydrolysate of the ampoule method, which gave a more complete breakdown, was distilled in vacuum at 50°C to remove the acid, then washed twice with water, concentrated and chromatographed as described above. A qualitative and rough quantitative comparison of the free and bound amino acids is given in Table II.

TABLE II

Results of amino acids chromatography

Name of acid	Free (soluble) amino acids	Bound amino acids
Alanine	+	+
Arginine	++	+
Aspartic acid	+	++
Cystine	—	+
Glutamic acid	+	++
Glycine	+	+
Lysine	+	+
Proline	++	+
Hydroxyproline	+	+
Serine	+	—
Tyrosine	—	+
Threonine	+	—
Histidine	++	+

Total and soluble nitrogen were determined by the Nessler and by the micro-Kjeldahl method. Both methods gave identical results. Total nitrogen of the whole pollen was 2.0%, and the soluble acids nitrogen was 0.81–0.83%.

Sugars were extracted as described by Given and Stranghen[6] the proteins precipitated with lead acetate and the excess of lead was precipitated with sodium carbonate. The sugars were identified by paper chromatography using water-butanol-acetic acid (1:4:1), as a developer and spraying with 1% *p*-anisidine hydrochloride. Quantitative determinations were made by the anthrone method according to Roe[7]. Reducing sugars were determined by the Somogyi-Nelson method. The results agree in general lines with the data published by Todd and Bretheric[3], Lunden[4] and Nilsson[5] for *P. silvestris* and *P. montana*. Qualitative and quantitative comparison between their and our results is given in Table III.

TABLE III

Sugar analysis in pollen of various species of Pinus in per cent

Sugar	*P. silvestris*[3]	*P. montana*[4]	*P. canariensis*
Total	8 — 14	11.5 — 12.5	11.7
Sucrose	11.0	9.0	9.7
Glucose	0.4	0.3	0.25
Fructose	0.5	0.7	0.8
Reducing sugars	0 — 2.0	1.05	1.57
Non-reducing sugars	8 — 12	10.5 — 11.5	10.1

As it is evident from Table I, the content of the ether soluble substances in the *P. canariensis* pollen comprises 9.8% of the fresh weight. 7.4% proved to be saponifiable; the unsaponifiable residue gave a positive result in the Salkovsky reaction for sterols. It contains substances soluble in acetone and in 2N alkali.

The quantitative results for the ether soluble substances of our experiments fall within the results of Todd and Bertheric[3] entomophilous pollen (1–20%) and of Sosa-Bourdouil and Sosa[8] for anemophilous pollen (11%).

The main difference between the composition of the *P. canariensis* pollen and the pollen of the other species of Pinus seems to lie in the abundance of the free proline in the first. A correlation between proline content in the pollen and the water content of leaves was shown by Kamble and Macpherson[9] for rye. There the amount of proline increased in the course of wilting and the authors suggest that it is synthesized from glutamic acid. A similar correlation may be suggested for *P. canariensis* as the water content of leaves of this species (6%) varies considerably from the water content of the leaves of other species (30%).

Another difference between the *P. canariensis* and other species is the ratio between the free basic amino acids in pollen and in the leaves. In entomophilous plants this ratio in pollen does not differ significantly from the ratio in the leaves, but the water content of the two also do not differ significantly. In Pinus canariensis the quantity of the free amino acids in pollen as found here exceeds considerably their amounts in leaves as reported by Virtanen[1].

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